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A STUDY OF PRESYNAPTIC SLOW POTENTIALS IN VIRUS-INFECTED SYMPATHETIC GANGLIA OF THE RAT

BY J. DEMPSHER AND J. ZABARA

From the Laboratories of Pharmacology and the Institute of Neurological Sciences, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, U.S.A.

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In order to explain the occurrence of spontaneous impulses, originating in presynaptic nerve endings and discharged simultaneously and synchronously in pre- and post-ganglionic nerves of rat sympathetic ganglia infected with pseudo-rabies virus, the following working hypothesis was adopted. Spontaneous impulses originated in presynaptic nerve endings because the virus infection interfered with the normal action of an inhibiting system having its origin in the central nervous system (C.N.S.) and exerting its action upon the presynaptic nerve endings through the release of substances such as gamma aminobutyric acid, adrenaline, and noradrenaline. Interference with the normal action of the inhibitory system resulted in a spontaneous release of acetylcholine (ACh), which stimulated the presynaptic nerve endings to initiate the discharge of impulses in both nerves, antidromic in the preganglionic nerve, orthodromic in the postganglionic nerve (Dempsher, Larrabee, Bang & Bodian, 1955; Dempsher & Riker, 1957; Dempsher, Tokumaru & Zabara, 1959).

An inherent weakness of the working concept presented above has been that, although based on experiments in which we used electrophysiological techniques, no electrical event had been described which could actually be located in the presynaptic nerve endings, the suggested site of interaction of excitatory and inhibitory systems. The purpose of this paper is to report experiments supporting the suggestion that the electrical event in the presynaptic nerve endings accompanying interaction of excitatory and inhibitory systems in virus-infected rat sympathetic ganglia is a slowly changing, decreasing potential change, the presynaptic slow potential.

METHODS

Preparation of the virus inoculum to infect the rat superior cervical ganglion was described in detail in previous publications (Tokumaru, 1957; Dempsher *et al.* 1959). Briefly, rat superior cervical ganglia were infected following intraocular inoculations of 0·05 ml. of undiluted suspension of L strain pseudo-rabies virus harvested from infected

cultures of monkey and rabbit kidney cells. Two methods were used to observe function in nerves of excised infected sympathetic ganglia. In the first method, impulses in pre- and post-ganglionic nerves, or from two different points on the preganglionic nerve, were picked up by platinum electrodes and recorded simultaneously by means of condenser-coupled pre-amplifiers and a double beam cathode-ray oscillograph. The condenser-coupled pre-amplifiers were set to have a constant amplification factor from 0·5 to 500 c/s and down one half at 3·5 kc/s. This method was used, and believed adequate, only for detecting the presence or absence of slow potentials simultaneously at two different points in the preganglionic nerve, or simultaneously in both pre- and post-ganglionic nerves. A check with a direct-coupled pre-amplifier and Ag–AgCl electrodes showed that slow potentials of approximately 0·5 sec duration or less were not significantly altered by platimum electrodes and condenser-coupled amplification maintained at the above settings. On the other hand, observations of the form of, and effect of drugs upon, the slow potential were carried out by means of Ag–AgCl electrodes, a low-gain direct-coupled pre-amplifier, and a single beam oscilloscope containing a high-gain direct-coupled amplifier. In both methods the excised preparation was maintained in a bathing solution (Dempsher *et al.* 1955) equilibrated with a mixture of 95 % oxygen and 5 % CO_2.

The experiments deal with the application to infected ganglia of ACh, physostigmine, gamma aminobutyric acid, adrenaline, noradrenaline, tubocurarine, and thiosemicarbazide. All concentrations of these drugs refer to the final concentration of the salt in the bathing solution.

RESULTS

Characteristics of the slow potential. When virus-induced activity was observed, two kinds of potential changes were observed: a slowly changing action potential (hereafter referred to as the slow potential), on which was superimposed a rapidly changing action potential (hereafter referred to as the spike potential) (Fig. 1). Whenever virus-induced activity appeared in both nerves, slow potentials were observed to be discharged simultaneously and synchronously in pre- and post-ganglionic nerves (Fig. 1).

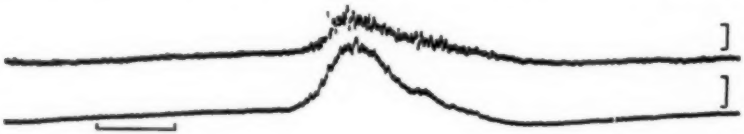

Fig. 1. Slow potentials with superimposed spikes recorded by means of condenser-coupled pre-amplifiers with wide frequency responses from an excised superior cervical ganglion infected with pseudo-rabies virus. Upper oscillogram from preganglionic, lower oscillogram from post-ganglionic trunk. Upper perpendicular potential scale applies to upper oscillogram, lower perpendicular potential scale to lower oscillogram; both show 100 μV. Horizontal time scale shows 200 msec.

The slow potential in the preganglionic nerve was still present in advanced infections even if the slow potential in the post-ganglionic nerve was absent, and even if electrical stimulation of the pre-ganglionic nerve failed to evoke a post-ganglionic response (Fig. 2). The magnitude of the slow

potential in the preganglionic nerve was greatest when recorded near the ganglion; a decrement occurred as the electrodes were moved along the preganglionic nerve away from the ganglion (Fig. 3). With DC recording the form of the slow potential in the preganglionic nerves was seen to vary from a single wave 250 msec in duration to multiple waves of one or

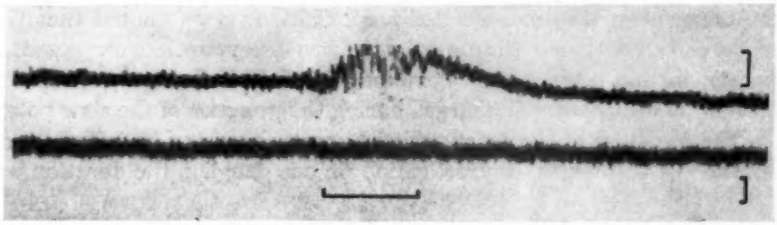

Fig. 2. Slow potential with superimposed spikes recorded by means of condenser-coupled pre-amplifiers with a wide frequency response from an excised rat superior cervical ganglion in an advanced stage of infection by pseudo-rabies virus. Upper oscillogram from preganglionic, lower oscillogram from post-ganglionic trunk. Upper perpendicular potential scale showing 10 μV applies to upper oscillogram, lower perpendicular potential scale showing 5 μV applies to lower oscillogram; horizontal time scale shows 200 msec.

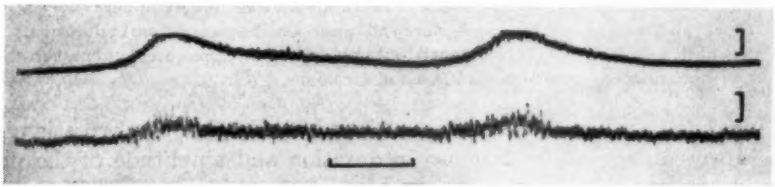

Fig. 3. Slow potentials and superimposed spikes recorded simultaneously by means of condenser-coupled pre-amplifiers with a wide frequency response from two positions on the preganglionic trunk of an excised rat superior cervical ganglion infected with pseudo-rabies virus. Upper oscillogram from preganglionic trunk 1·5 mm from the preganglionic pole of the ganglion; lower oscillogram from preganglionic trunk 6·5 mm from preganglionic pole of the ganglion. Upper perpendicular potential scale showing 20 μV applies to upper oscillogram, lower perpendicular potential scale showing 10 μV applies to lower oscillogram; horizontal time scale shows 200 msec.

more seconds in duration (Fig. 4, upper oscillogram). As long as the slow potential remained above a resting level, spike potentials continued to be discharged (Fig. 4).

Physostigmine. It has previously been concluded (Dempsher & Riker, 1957) that physostigmine produced a simultaneous and synchronous increase followed by a decrease in virus-induced activity in both nerves of rat sympathetic ganglia infected with pseudo-rabies virus by exerting its

effects upon the site of origin (pace-maker) in the presynaptic nerve endings. In accord with this view was the increase in activity produced by physostigmine in preganglionic nerves in infections so advanced that there was no spontaneous activity in the post-ganglionic nerves, nor could activity be evoked in those nerves by preganglionic excitation (Dempsher & Riker, 1957). When the effects of physostigmine 10 μg/ml. were observed with direct-coupled amplification, it was noted that this drug greatly prolonged the duration of the presynaptic slow potential (Fig. 4); in one ganglion the duration was increased from 3 to 26 sec. Spikes were continually discharged during the presence of the slow potential. The duration of the quiet period between bursts of activity was reduced in the presence of physostigmine; in one ganglion the duration was

Fig. 4. Effects of physostigmine 10 μg/ml. on slow potential and superimposed spikes in preganglionic trunk of rat superior cervical ganglion infected with pseudo-rabies virus. Perpendicular potential scale showing 100 μV applies to both oscillograms; horizontal time scale shows 200 msec. Oscillogram, 12 sec in duration, has been removed to fit slow potential into photograph. Upper oscillogram, control; lower, after physostigmine has acted for 30 sec.

reduced from 2·1 to 0·1 sec. On continued application of physostigmine there was a progressive decrease in duration and amplitude of the presynaptic slow potential until it disappeared. At no time were spike potentials observed in the absence of the slow potential.

Acetylcholine. The effects of ACh 100–200 μg/ml. on the presynaptic slow potential were similar to those produced by physostigmine. The duration of the presynaptic slow potential was greatly prolonged and the duration of the quiet period between bursts of activity was greatly reduced. For example, in one ganglion the duration of the slow potential was increased from 2·6 to 11·5 sec, whereas the duration of the quiet period was reduced from 3·0 to 0·5 sec. As in the case of physostigmine, continued application of ACh resulted in a progressive decrease in duration and amplitude of the presynaptic slow potential until it disappeared. Spike potentials were observed only whenever slow potentials were present.

Tubocurarine. It has been concluded (Dempsher & Rike, 1957) that tubocurarine exerted actions upon presynaptic nerve endings of rat sympathetic ganglia because it depressed simultaneously and synchronously virus-induced activity in both nerves, and in preganglionic nerves

in infections so advanced that activity could not be evoked in post-ganglionic nerves by preganglionic excitation. When applied to virus-infected ganglia, tubocurarine 150 μg/ml. produced a progressive decrease in duration and amplitude of the presynaptic slow potential (Fig. 5).

Gamma aminobutyric acid, adrenaline, and noradrenaline. It has been suggested (Dempsher *et al.* 1959) that gamma aminobutyric acid, adrenaline, and noradrenaline exerted effects upon the presynaptic nerve

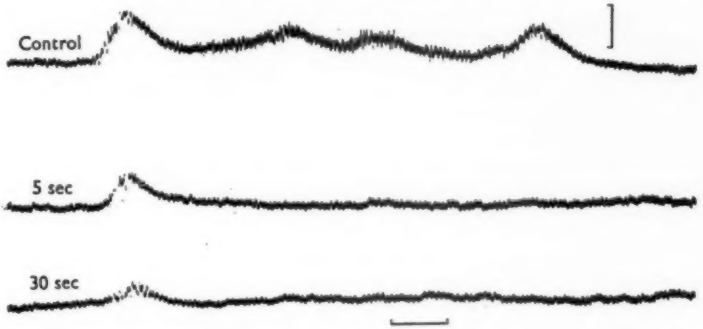

Fig. 5. Effects of tubocurarine 150 μg/ml. on slow potential and superimposed spikes in preganglionic trunk of rat superior cervical ganglion infected with pseudo-rabies virus. Perpendicular potential scale showing 100 μV applies to all oscillograms; horizontal time scale shows 200 msec. Top record, control; middle and lower after tubocurarine has acted for times shown.

endings because they altered virus-induced activity in both nerves simultaneously and synchronously, and because they depressed spontaneous activity in preganglionic nerves in infections so advanced that activity could not be evoked in post-ganglionic nerves by preganglionic excitation. When applied to virus-infected ganglia, gamma aminobutyric acid 1–10 μg/ml. produced a decrease in duration of the presynaptic slow potential (Fig. 6). The effects of adrenaline 1–50 μg/ml. and of noradrenaline 50 μg/ml. were similar to those of gamma aminobutyric acid, a decrease in duration of the presynaptic slow potential.

Thiosemicarbazide. It has been concluded (Dempsher *et al.* 1959) that thiosemicarbazide, known to interfere with the synthesis of gamma aminobutyric acid, acted upon presynaptic nerve endings because it increased the activity in both nerves simultaneously and synchronously. When the effects of thiosemicarbazide 10 μg/ml. were observed with direct-coupled amplification, it was observed that this drug increased the duration of the slow potential from approximately 0·5 to 1·5 sec, and increased the amplitude from approximately 60 to 110 μV. The duration of the quiet period between bursts of activity was reduced in the presence

of thiosemicarbazide from approximately 1·5 to 0·8 sec. Spike potentials were observed only whenever slow potentials were present.

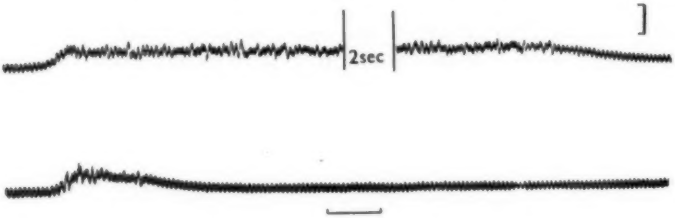

Fig. 6. Effects of gamma aminobutyric acid 1–10 μg/ml. on slow potential and superimposed spikes in preganglionic trunk of rat superior cervical ganglion infected with pseudo-rabies virus. Perpendicular potential scale showing 100 μV applies to both oscillograms; horizontal time scale 200 msec. Small regular waves on base line are 60 cycle interference. Upper record control; lower, after GABA has acted for 10 min.

DISCUSSION

The present observations suggest the possibility that the electrical manifestation of the interaction of excitatory and inhibitory systems in the presynaptic nerve endings is a slow potential. In support of this view is the presence in the preganglionic nerve of a slow potential with superimposed spikes. The most likely sites of origin of this slow potential appear to be the presynaptic nerve endings since its magnitude is greatest nearest the ganglion, and it is present in advanced infections where the post-ganglionic nerves are neither spontaneously active, nor can be excited preganglionically. Slow potentials originating in presynaptic nerve endings have not been commonly observed. Barron & Matthews (1938) described slow dorsal root potentials in cats, frogs, and monkeys and concluded that the slow potentials had their origin in the terminal portions of the afferent neurone, i.e. the presynaptic nerve endings. Because antidromic spikes were discharged only and continually, during the presence of the presynaptic slow potential, it seems reasonable to suggest that the slow potential described in the present paper represents a progressive depolarization of the presynaptic nerve endings which serves as a focus for depolarization of the preganglionic axon. The spikes appeared superimposed upon the slow potential because observations of both potentials were made usually with recording electrodes near the ganglion, where the magnitude of the slow potential was greatest. A similar explanation was given earlier by Adrian (1931) for the relationship between slow potentials and superimposed spikes discharged spontaneously in abdominal ganglia and their peripheral nerves of *Dytiscus marginalis*; in this instance it was suggested that the slow potential represented a progressive depolarization of den-

drites which served as a focus for depolarization of the peripheral nerves. More recently Eccles (1943) and Laporte & Lorente de Nó (1950) have suggested that slow potentials observed in sympathetic ganglia of the cat and turtle represent depolarization of the somata of post-ganglionic cells and serve as a focus for initiating impulses in post-ganglionic axons.

The increase in size of the presynaptic slow potential by physostigmine and the decrease in size by tubocurarine suggest that the role of ACh at presynaptic nerve endings is to increase excitability by increasing the duration of the slow potential. In accord with this suggestion was the increase in duration of the slow potential whenever ACh was applied to infected ganglia. Similar effects of physostigmine and tubocurarine on motor end-plate potentials had been observed earlier by Eccles, Katz & Kuffler (1941, 1942), and on miniature end-plate potentials more recently by Fatt & Katz (1952). The decrease in size of the presynaptic slow potential, with consequent decrease in the number of spikes, whenever possible inhibitors, gamma aminobutyric acid, adrenaline, and noradrenaline were applied, suggests that these agents depress excitability by decreasing the size of the slow potential. In accord with this view was the increase in the duration and amplitude of the presynaptic slow potential whenever thiosemicarbazide was added. Thus it would seem reasonable to suggest that the size of the slow potential at any one time represents a finely balanced antagonism of excitatory and inhibitory components.

In order to explain the new findings described above, the previous working hypothesis is extended as follows: spontaneous impulses originate in presynaptic nerve endings of rat sympathetic ganglia infected with pseudo-rabies virus because the virus infection interferes with the normal action of an inhibiting system having its origin in the C.N.S. and exerting its action upon the presynaptic nerve endings through the release of substances such as gamma aminobutyric acid, adrenaline, and noradrenaline; interference with the normal action of the inhibitory system results in the spontaneous release of ACh which stimulates the presynaptic nerve endings to initiate the discharge of impulses in both nerves, antidromic in the preganglionic nerve, and orthodromic in the post-ganglionic nerve. The electrical event accompanying the interaction of excitatory and inhibitory systems at the presynaptic nerve endings is a slowly changing, decreasing potential change, the presynaptic slow potential.

SUMMARY

1. By means of standard electrophysiological methods, two kinds of spontaneous potential changes were observed in excised rats' superior cervical ganglia following infection with pseudo-rabies virus, a slowly

changing action potential, the slow potential, on which was superimposed a rapidly changing action potential, the spike.

2. When virus-induced activity appeared in both nerves, slow potentials were observed to be discharged simultaneously and synchronously in pre- and post-ganglionic nerves.

3. The slow potential in the preganglionic nerve was still present in advanced infections when the slow potential in the post-ganglionic nerve was absent, and when electrical stimulation of the preganglionic nerve failed to evoke a post-ganglionic response. The magnitude of the presynaptic slow potential was greatest when recorded near the ganglion and a decrement occurred as electrodes were moved along the preganglionic nerve.

4. Spikes continued to be discharged in the preganglionic nerve so long as the presynaptic slow potential remained above a resting value.

5. Physostigmine and ACh initially increased the size of the presynaptic slow potential, then decreased it. Thiosemicarbazide increased the size of the presynaptic slow potential. Tubocurarine, gamma aminobutyric acid, adrenaline, and noradrenaline decreased the size of the presynaptic slow potential.

6. It is suggested that the slow potential located in the presynaptic nerve endings served as a focus for depolarization of the preganglionic axon, and represented the electrical event accompanying interaction between excitatory and inhibitory systems at the presynaptic nerve endings.

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CENTRAL CONTROL OF SYMPATHETIC CARDIO-ACCELERATION IN THE CAT

BY C. N. PEISS*

From the Department of Physiology, Stritch School of Medicine and the Graduate School, Loyola University, Chicago, Illinois, U.S.A.

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At the present time there is little conclusive evidence as to the location of a cardioaccelerator centre. It is a curious fact that the extensive series of investigations which have provided comprehensive information about the vasomotor centre (Scott & Roberts, 1923; Scott, 1925; Monnier, 1939; Wang & Ranson, 1939*a*; Alexander, 1946; Bach, 1952) has failed to clarify the anatomical basis for a medullary cardioaccelerator centre. In an early text-book of physiology (Howell, 1897) the statement is made that 'the situation of the centre for the augmentor nerves of the heart is not definitely known, although from analogy it seems probable that it will be found in the bulb'. It appears that current teaching about the cardio-accelerator centre is still based on analogy to a large extent. Aside from specific text-book descriptions of the location of the cardioaccelerator centre in the medulla close to the vasomotor centre, there are few reports in the research literature of this type. In reviewing the cardioaccelerator mechanism, Bard (1929) concluded that the centre is probably in the medulla oblongata. The chief basis for this conclusion appears to be the indirect evidence obtained from studies on heart-rate changes reflexly induced through the inhibitory and accelerator nerves (Hunt, 1899; Bayliss, 1908; von Brücke, 1917; McDowall, 1931, 1938; Bronk, Ferguson & Solandt, 1934). These papers are the chief basis for the concept of reciprocally innervated cardiac centres. It has also been stated (Bard, 1929) that Ranson & Billingsley (1916) observed cardiac acceleration during electrical stimulation of the floor of the fourth ventricle after section of the vagi. In fact, the paper of Ranson & Billingsley recorded no such result. It is a well documented fact that reflex activation of the vasomotor centre is associated with cardiac acceleration. This fact, coupled with the known location of the vasomotor and cardio-inhibitory centres in the medulla, makes the postulate of a medullary cardioaccelerator centre in close proximity to the vasomotor centre a most attractive and reasonable hypothesis.

* John and Mary Markle Scholar in Medical Sciences.

Careful control of experimental procedures is necessary to test this hypothesis properly. There are four ways by which heart rate may be increased during electrical stimulation of the brain: (1) activation of sympathetic outflow to the heart; (2) central inhibition of vagal tone; (3) release of epinephrine and norepinephrine from the adrenal medulla; and (4) certain non-specific factors relating to the metabolic status of the heart.

Failure to evaluate these factors adequately has contributed to the uncertainty with regard to the cardioaccelerator centre. The conclusion that cardioaccelerator fibres are being activated is valid only when there is complete assurance that the other three mechanisms are inoperative. Once this point is reached, it must still be shown whether the fibres being stimulated in the brain are afferent or efferent. It is the purpose of this paper to present such a controlled analysis of the postulated medullary cardioaccelerator centre.

METHODS

The data in this paper were obtained from ninety-six young adult cats, of which fifty-five were anaesthetized with sodium pentobarbital 30 mg/kg intraperitoneally. The remaining forty-one animals were anaesthetized with alpha-chloralose 70–100 mg/kg intravenously. The differences in cardiovascular responses obtained from the two groups are discussed in detail below. Blood pressure was recorded from a carotid artery with a Statham P23Db transducer, Sanborn carrier wave amplifier and optical galvanometer. The pressure recording system was filled with heparin to prevent coagulation of the blood. Heart rate was recorded continuously with an integrating cardiotachometer (McCook & Peiss, 1959). Specific latency of changes in heart rate induced by electrical stimulation was determined by pulse-to-pulse measurements from high-speed recordings. Stimulation parameters were 2–7 V, 2 msec pulse duration and a frequency of 70 c/s. These parameters have been shown to be optimum for eliciting cardiovascular responses from the brain stem (Peiss, 1956). The positions of knife transections, hypothalamic lesions and stimulating electrodes were verified histologically from serial sections of the brain stem.

All animals were bilaterally vagotomized by dividing the vagosympathetic nerves high in the neck. This eliminated changes in vagal tone as a possible source of cardioacceleration. Increases in heart rate due to activation of the adrenal medulla were ruled out by the requirement that only cardioaccelerator responses which occurred with a latency of less than 6 sec would be considered as valid primary accelerator responses. The role of the adrenal medulla is shown below in the Results section. Since the metabolic status of the heart will have important consequences on its dynamic performance, we have eliminated from consideration any animals whose mean blood pressure was below normal limits. This was done solely to eliminate as far as possible non-specific factors (item 4 above) affecting the heart rate. It is conceivable that the blood-pressure rise following stimulation, with an attendant increase in coronary flow, might result in appreciable changes in heart rate in an animal with poor coronary circulation.

RESULTS

Areas stimulated in the medulla

There is general agreement that the vasomotor centre is located in the lateral reticular formation, extending dorsally to the floor of the fourth

ventricle (cf. Wang, 1955). It is our experience that the greatest pressor responses are obtained from an area extending 1–5 mm rostral to the obex, 1–3 mm to either side of mid line and from 0 to 3 mm ventral to the floor of the fourth ventricle. Although responses can be obtained from a much wider area than that outlined above, they generally are smaller in magnitude and less consistent from animal to animal. Whether this area of greatest responsiveness constitutes the integrative centre is not known. It is sufficient for our purposes to point out that large increases in mean pressure continue to be elicited from this region after mid-collicular transection, indicating that this area does not merely represent a site of afferent input to higher levels of the central nervous system. The picture is further complicated by the fact that an appreciable part of the blood-pressure rise resulting from stimulation of this area of the medulla is due to the activation of augmentor pathways to the heart (Peiss, 1958).

In these experiments the ventrolateral portion of the medulla has also been explored. Stimulation of this area, however, may not involve activation of a medullary vasomotor mechanism, but rather of efferent pathways from the hypothalamus (Magoun, Ranson & Hetherington, 1938; Wang & Ranson, 1939*b*). Since stimulation of the hypothalamus results in large increases in heart rate mediated through the cardiac sympathetics (Beattie, Brow & Long, 1930; Manning & Peiss, 1958, 1960; Rushmer, Smith & Franklin, 1959), it is vital to differentiate these two regions of responsiveness in the medulla. Cardioacceleration resulting from activation in the medulla of hypothalamospinal pathways affords no evidence for a medullary cardioaccelerator centre. Such inferences have been made in the older literature. In this paper, therefore, we shall distinguish between 'dorsal' and 'ventrolateral' medullary areas.

Responses under pentobarbital anaesthesia

Short-latency responses. Over one thousand stimulations of the dorsal medulla in fifty-five cats resulted in only a few clear-cut cardiac accelerations that could probably be ascribed to activation of sympathetic outflow to the heart. The heart-rate increases were never more than 15% of the pre-stimulation level. Since both vasomotor and augmentor responses could easily be elicited under this mode of anaesthesia (Peiss, 1958), it appeared reasonable to assume that a medullary cardioaccelerator centre would also be operative if it existed. Moreover, if the postulated close proximity of the medullary cardioaccelerator centre to the vasomotor centre, and its reciprocal relation to the cardio-inhibitory centre, were real, it appeared reasonable that we should have succeeded in activating it in the area of the medulla which yielded maximal vasoconstrictor and augmentor activity. The fact that the accelerator responses were rarely

evoked casts doubt on the postulated location of the cardioaccelerator centre.

On the other hand, in animals under the same anaesthesia it is common to obtain significant short-latency acceleration by stimulation of the ventrolateral medulla. It is our experience that these areas are usually devoid of the very large pressor responses observed when the dorsal medulla is

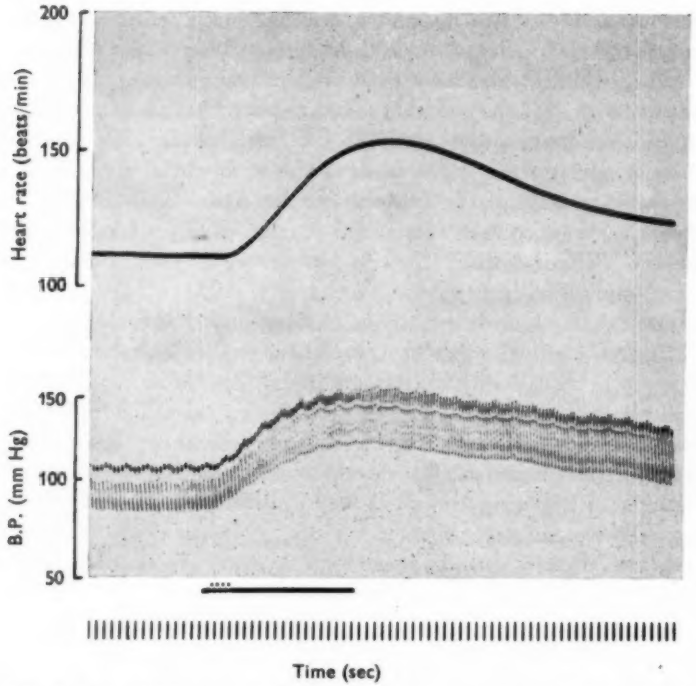

Fig. 1. Short-latency cardioaccelerator response to stimulation of ventrolateral medulla in the cat under pentobarbital anaesthesia. Stimulation parameters: 3·2 V, 2 msec, 70 c/s. Solid bar indicates period of stimulation.

stimulated. Figure 1 shows an example of a short-latency response from the ventrolateral medulla in a cat under pentobarbital. The electrode was located at the extreme border of the lateral reticular formation near the vestibulospinal tract. In other cats similar responses were obtained from the area of the ventral reticulospinal tract.

Long-latency responses. Stimulation of the dorsal medulla frequently elicited cardiac acceleration after a latency of 12–20 sec from the onset of stimulation. Figure 2 illustrates such a response, from a point 1·5 mm below the floor of the fourth ventricle, 2 mm right of mid line and 2 mm

rostral to the obex. Despite the fact that pressor and augmentor activity is observed within 5 sec, acceleration does not occur until 12·5 sec after onset of stimulation. It would be presumptive to assume that this is the result of peripheral delay at the effector sites on the heart, especially since cardioaccelerator responses obtained by hypothalamic stimulation have latencies of 1–5 sec. The delayed acceleration is most probably due to

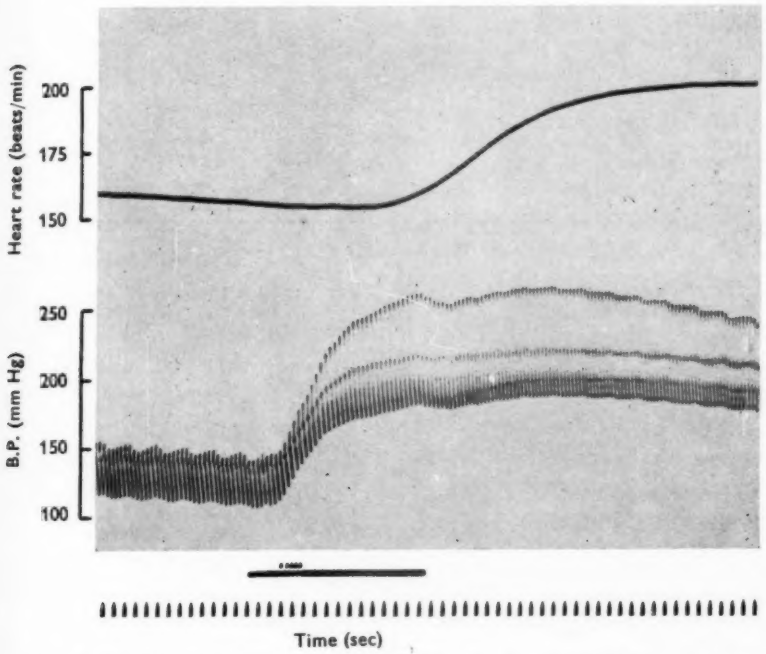

Fig. 2. Long-latency cardioaccelerator response to stimulation of dorsal medulla in the cat under pentobarbital anaesthesia. Stimulation parameters: 2·6 V, 2 msec, 70 c/s. Solid bar indicates period of stimulation.

activation of the adrenal medulla and subsequent arrival at the heart of its secretion. This possibility was tested in a series of seven animals, all of which showed delayed acceleration similar to that shown in Fig. 2. These seven cats were then subjected to acute bilateral adrenalectomy and restimulated at the same point in the medulla and at the same parameters of stimulation. The results of a typical experiment are shown in Fig. 3. It is apparent that most of the accelerator (and augmentor) response was eliminated by adrenalectomy, which supports our ideas concerning the origin of these long-latency accelerator responses. The decreased rise in blood pressure after adrenalectomy is due, in part, to elimination of the

accelerator and augmentor responses. In other animals most of the pressor response remained after adrenalectomy.

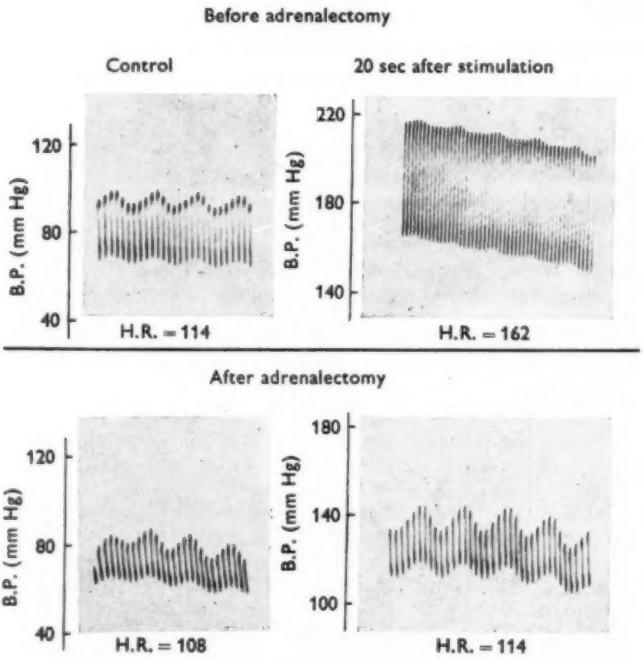

Fig. 3. Effect of bilateral adrenalectomy on long-latency cardioaccelerator response to stimulation of dorsal medulla in the cat under pentobarbital anaesthesia. Stimulation parameters: 3 V, 2 msec, 70 c/s; H.R., heart rate (beats/min).

Responses under chloralose anaesthesia

In striking contrast to results obtained with cats under pentobarbital, it was found that large increases in heart rate, with latencies of 1–5 sec, result when the dorsal medullary area is stimulated in cats under chloralose anaesthesia. A typical response is shown in Fig. 4. Although cardioacceleration is not obtained as consistently as augmentation and vasoconstriction, adequate exploration of the dorsal medullary area outlined above is usually successful in locating an active region in most cats. Moreover, increases in heart rate are consistently larger than those seen under pentobarbital, amounting to as much as an 80% increase over the prestimulation level. This greater response is not due to differing control levels of heart rate in the two sets of animals under different anaesthetics.

Heart rates in both series, after vagotomy, were in the range of 100–175 beats/min.

The question now arises as to the cause of the difference between cardioaccelerator responses to electrical stimulation of the dorsal medulla under the two anaesthetics. If the postulated existence and location of a medullary cardioaccelerator centre is valid, one must conclude that there is a

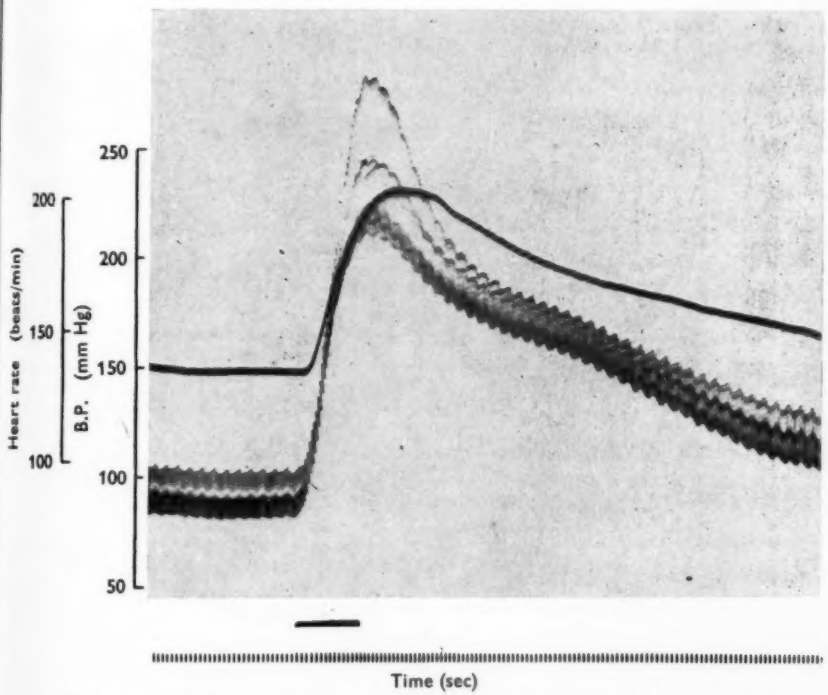

Fig. 4. Short-latency cardioaccelerator response to stimulation of dorsal medulla in the cat under chloralose anaesthesia. Stimulation parameters: 1·9 V, 2 msec, 70 c/s. Solid bar indicates period of stimulation.

differential effect of barbiturate on those cells and/or fibres subserving acceleration of the heart as compared with those subserving augmentation of myocardial contractile force and blood vessel constriction. This does not appear to be a reasonable assumption.

It has been shown previously (Manning & Peiss, 1958) that usual anaesthetic doses of pentobarbital severely depress or eliminate cardiovascular responses to electrical stimulation of the hypothalamus. On the other hand, these responses from the hypothalamus are readily elicited

under chloralose. A more logical conclusion from the above data is, therefore, that the accelerator area in the dorsal medulla, which is quite responsive under chloralose, is in fact an afferent pathway to higher levels of the central nervous system. If the fibres from this area relay in the hypothalamus, it might then be expected that the response to dorsal medullary stimulation would be blocked or severely depressed under pentobarbital anaesthesia.

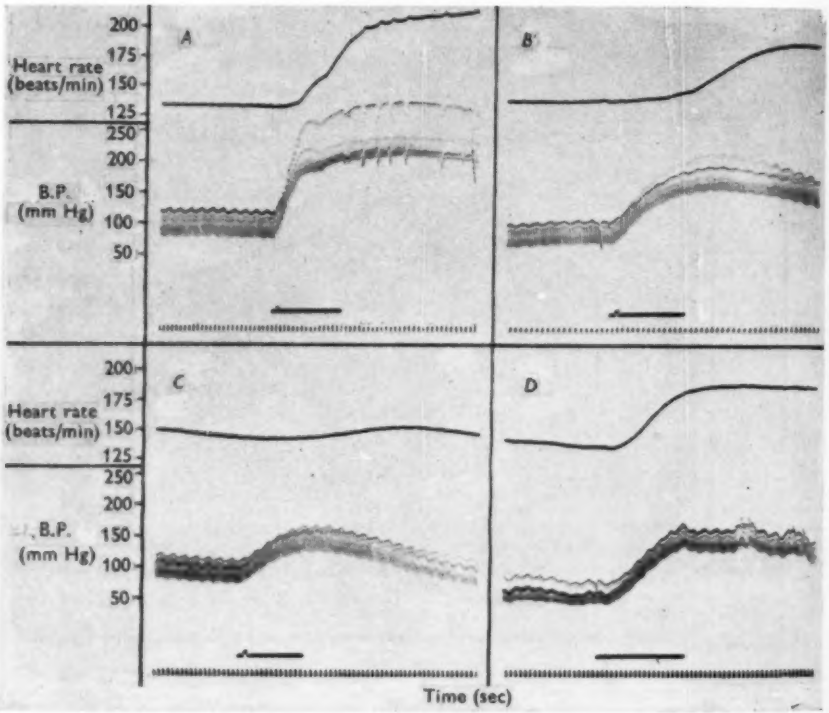

Fig. 5. Effect of lesions (just caudal to hypothalamus) on cardioaccelerator response to stimulation of dorsal medulla in the cat under chloralose anaesthesia. *A*. Pre-lesion response. *B*. Response after partial lesion (see text for details). *C*. Response after more extensive lesion (see text for details). *D*. Accelerator response from ventrolateral medulla after above lesion. All stimuli at 2·4 V, 2 msec, 70 c/s. Solid bars indicate periods of stimulation.

Figure 5 illustrates an experiment testing this critical point as to whether or not cardioacceleration following stimulation of the dorsal medullary area is due to stimulation of afferents to higher levels of the central nervous system. In Fig. 5*A*, a point in the dorsal medulla was stimulated

in a vagotomized cat under chloralose anaesthesia. The point stimulated was 1 mm below the floor of the fourth ventricle, 1·5 mm left of mid line and 2·5 mm rostral to the obex. Within 3 sec, heart rate, pulse pressure and mean pressure increased. At the peak of the response heart rate had increased from 134 to 220 beats/min, pulse pressure from 46 to 82 mm Hg, and mean pressure from 100 to 250 mm Hg. The recording shown in Fig. 5*B* was made after partial destruction of the brain stem with electrolytic lesions. A series of five electrodes were placed at 2 mm intervals across the brain stem. Each electrode carried a current of 5 mA for 20 sec. Since more extensive lesions were made subsequently, it is possible only to estimate the extent of the lesion present when the record shown in Fig. 5*B* was made. From the data of Carpenter & Whittier (1952), it is estimated that the lesion extended laterally on each side of the mid line to the nucleus ventralis posteromedialis, dorsally to a level at the dorsal border of the red nucleus and ventrally to a level at the ventral border of the red nucleus, involving a segment of the brain stem 10 mm wide, 2 mm in the dorsoventral plane and 1 mm in the rostrocaudal plane. The stimulus strength in Fig. 5*B* was the same as in Fig. 5*A*. It is apparent that the magnitude of response was greatly diminished by the lesion, and that the latency for cardioacceleration was greatly increased. Figure 5*C* shows a recording made some minutes later, after further electrolytic destruction of the brain stem. Histological examination of a cross-section at the level of the red nucleus revealed practically total destruction of the ventral two-thirds of the brain stem, extending approximately 5 mm to each side of the mid line and dorsally to a level at the dorsal border of the nucleus centralis. The recording indicates that the same stimulus which was applied to the same point in the dorsal medulla in Fig. 5*A* now produces a modest rise in mean blood pressure, practically no change in pulse pressure and very little cardioacceleration. It appears that the pathways which influence cardiac rate and contractility are no longer operative. It is presumed that the rise in diastolic pressure indicates the integrity of a vasomotor pathway. The integrity of the vasomotor reflex arc has been demonstrated in other experiments by using bilateral carotid artery occlusion. Figure 5*D* shows the response in the same animal to stimulation in the ventrolateral medulla shortly after the record in Fig. 5*C* was obtained. An appreciable acceleration of the heart resulted, after a latency of 3·5 sec. This response was similar to that shown in Fig. 1, which was recorded from another cat with the electrode in approximately the same position. The response in Fig. 5*D* also confirms that the trauma resulting from electrolytic destruction of the brain stem did not affect the peripheral mechanisms involved in the cardioaccelerator response. Two other lesion experiments were performed with the same results.

Similar experiments have been performed by knife transections of the brain stem at the mid-collicular level. In seven experiments of this type, transection resulted in complete loss of the cardioaccelerator response to dorsal medullary stimulation in four cats. The other three cats showed 80–90 % loss of response. In all animals the transections were accomplished without appreciable fall in mean blood pressure.

DISCUSSION

Taken as a whole, the data in this paper provide little evidence for the existence in the dorsal medulla of an area involved in the integration of sympathetic cardioacceleration. The postulated spatial proximity of a cardioaccelerator centre to the medullary vasomotor centre appears to have little basis in fact. Under pentobarbital anaesthesia the only region in the medulla which gives rise to fairly consistent short-latency cardiac acceleration, when stimulated electrically, is the ventrolateral area, which is usually devoid of great pressor activity. It is most probable, therefore, that these ventrolateral areas of the medulla represent the site of hypothalamo-spinal pathways. This view is supported by the loss of reflex acceleration in vagotomized animals induced by bilateral carotid artery occlusion following mid-brain transections (T. K. Akers & C. N. Peiss, unpublished results).

In cats under chloralose anaesthesia stimulation of the vasomotor area of the dorsal medulla gives relatively consistent short-latency accelerator responses, in contrast to the almost complete absence of these responses under full anaesthetic doses of pentobarbital. If the responses under chloralose were due to activation of pathways to higher levels of the central nervous system, this anaesthetic difference would be consistent with previous findings that the hypothalamus is severely depressed by the barbiturates (Manning & Peiss, 1958). Evidence supporting the hypothesis that the dorsal medulla contains afferent pathways involved in sympathetic cardioacceleration is provided by the loss of most of the accelerator responses to dorsal medullary stimulation following mid-collicular transection or massive electrolytic lesions in the brain just caudal to the hypothalamus. Hunt (1899) noted many years ago that the 'accelerator centre' is resistant to influences which depress the vasomotor centre. One of the influences was curare. We have shown recently (Peiss & Manning, 1959) that curare has a direct depressant effect on the excitability of the medullary vasomotor centre, with little or no effect on areas in the hypothalamus which are involved in cardiovascular control. These findings are consistent with an anatomical separation of cardioaccelerator and medullary vasomotor areas of integration. These findings do not exclude the possibility of some integrative activity in the bulb with regard to sym-

pathetic cardioacceleration. The 10–15% increases in heart rate in the cats under pentobarbital, as well as the persistence of up to 20% of the response under chloralose after transection, supports the view that there may be such bulbar integration. However, until all the hypothalamic pathways to the heart are known it is impossible to determine whether or not such residual acceleration is due to efferent pathways from the hypothalamus. The findings indicate that, of the sympathetic cardioacceleration that can be induced in a chloralosed cat, the great majority is dependent upon the integrity of brain-stem areas above the medullary vasomotor area. In the dog it has been shown that some cardiac acceleration occurs in a vagotomized, adrenalectomized, mid-brain-transected preparation following sciatic nerve stimulation (Chen, Lim, Wang & Yi, 1937). The heart rate in this experiment increased from a control level of 204 to a maximum response of 230 beats/min after stimulation.

The location of the integrative areas for sympathetic cardioacceleration in the cat are not known, although the bulk of the evidence points to the hypothalamus for one. This is well documented by the studies of Beattie *et al.* (1930), Manning & Peiss (1958, 1960) and Rushmer *et al.* (1959).

The experiments presented here seem to indicate that the over-all control of heart rate is integrated at several levels, the vagal nucleus in the medulla and at least one higher level, probably hypothalamic. The fact that various reflex responses induce slowing or acceleration of the heart by reciprocal inhibition has been known for many years (cf. McDowall, 1938). The experiments reported here indicate, therefore, that the afferent nerves involved in such reflex responses send off collaterals, at several levels of the central nervous system, to those areas involved in sympathetic and parasympathetic control of heart rate. It is not known whether the process of reciprocal inhibition operates in all reflexes affecting heart rate. In so far as the bulbar mechanism is concerned, it appears that change in vagal discharge is the primary mechanism. Sympathetic control of heart rate seems to operate chiefly at higher levels of the central nervous system.

In our experiments on cats under chloralose anaesthesia it was found that augmentor responses are elicited much more consistently than accelerator responses by stimulation of either the dorsal medulla or the hypothalamus. It is a reasonable assumption that the number of sympathetic fibres innervating the myocardium (augmentor fibres) is much in excess of the number of fibres innervating nodal tissue (accelerator fibres). If this is the case, one might reasonably expect a quantitative difference in the number of fibres involved in the central representation of these peripheral sympathetic fibres.

SUMMARY

1. Stimulation of the dorsal medulla, in regions producing maximal vasoconstrictor and augmentor responses, produced little short-latency cardiac acceleration in vagotomized cats under pentobarbital anaesthesia. Long-latency (12–20 sec) cardioacceleration in these cats was largely eliminated after bilateral adrenalectomy.

2. Stimulation of ventrolateral areas of the medulla produced large short-latency increases in heart rate in vagotomized cats under pentobarbital anaesthesia, presumably through activation of hypothalamospinal pathways.

3. In vagotomized cats under chloralose anaesthesia, stimulation of the dorsal medulla results in large cardioaccelerator responses with latencies of 1–5 sec.

4. These responses are partly or wholly eliminated by electrolytic lesions in the brain stem just caudal to the hypothalamus or by midcollicular transections, indicating that they are due to activation of afferent pathways to higher levels of the central nervous system.

5. The results in this paper do not support the concept of integration of sympathetic cardioacceleration in the medulla of the cat.

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THE MODE OF ACTION OF HAGEMAN FACTOR IN THE RELEASE OF PLASMA KININ

By J. MARGOLIS

From the Institute of Pathology, Royal Alexandra Hospital for Children, Sydney, Australia

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A pain-producing and smooth-muscle-contracting substance, probably a polypeptide, is released from blood plasma by contact with glass and some other surfaces (Armstrong, Jepson, Keele & Stewart, 1957). In later work (Margolis, 1958*a*) well defined stages in the mechanism for the release of this plasma kinin have been described, and also the fact that kinin is not liberated from plasma which is congenitally deficient in Hageman factor (Ratnoff & Colopy, 1955). Hageman factor (HF) is not merely a hypothetical component whose presence is postulated to explain the steps of kinin formation, but is independently defined as the factor missing in a congenital coagulation abnormality known as the Hageman trait. Further, it is known that HF forms the connecting link between the changes involved in kinin release and those leading to blood coagulation and the formation of a permeability factor (Margolis, 1957, 1958*a*, 1959; Biggs, Sharp, Margolis, Hardisty, Stewart & Davidson, 1958; Hardisty & Margolis, 1959).

According to a concept formulated in a preliminary account (Margolis, 1958*b*), HF normally exists in the plasma in a biologically inactive state, that is, as a precursor. When plasma is brought into contact with glass and some other foreign surfaces (e.g. quartz, kaolin) HF is rapidly adsorbed by the surface, where it acquires enzymic activity and initiates the further stages of these reactions without itself returning into solution or being consumed in the process. In kinin release two such further stages have been recognized; they can be accounted for by the presence of at least two components which react successively and which have been provisionally designated components A and B. Because of the similarities in their functions and in some physical properties HF and component A were at first thought to be identical. Further work, however, has shown that this identification can no longer be maintained. Thus we can now distinguish three separate stages in the release of plasma kinin by contact with glass. The postulated order of these reactions is represented in Fig. 1. Activated

component A and plasma kinin are rapidly inactivated in plasma, but are relatively stable when partially purified. Components A and B are exhausted in the course of activation, component B being much more labile than A in this respect.

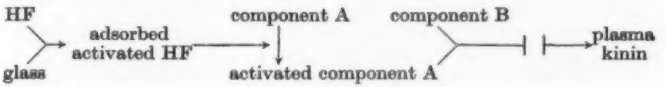

Fig. 1. Suggested mode of action of Hageman factor (HF); for details see text.

Unlike HF, which is defined by independent criteria, the other components are at present recognized only as functional entities. Something is known about their physical properties, such as heat stability, adsorbability and presence in crude protein fractions (Margolis, 1958*a*), but this information is still fragmentary and insufficient to characterize these factors as distinct substances. Future work may well reveal that each is really a complex system in itself.

The main object of this paper is to present evidence on the role of HF in the release of plasma kinin. The observations involving other components will be considered only in so far as they are necessary for the understanding of this initial stage of the process.

In the previous papers the ability of glass-activated preparations to release kinin, when added to intact plasma, was interpreted as being due to the formation of a 'contact factor' (equivalent to activated component A; see Fig. 1). This term will be avoided here because: (*a*) contact factor was at the time erroneously thought to be a direct activation product of HF, and (*b*) the term has since been justifiably used by other authors as a synonym of HF (Caen & Bernard, 1958; Soulier, Wartelle & Menache, 1958).

METHODS

Unless otherwise stated, all operations were performed in siliconed glassware. 'Intact' citrated human plasma and plasma derivatives were prepared by methods discussed in detail elsewhere (Margolis, 1957, 1958*a*).

HF-deficient (HF-dep) plasma was obtained for most of the experiments from a single patient with Hageman trait (cf. Biggs *et al.* 1958); identical results were obtained with fresh and lyophilized material. These were further confirmed on plasma from three other patients.

(1) *Plasma depleted of component B* (*B-dep plasma*) was prepared as follows. Intact plasma was rotated for 2 min with glass ballotini (microspherules 0·1 mm in diameter) 1 g/ml., i.e. about 200 cm² surface/ml. of plasma. It was then removed from the beads and immediately mixed with an equal volume of intact plasma. The mixture was incubated in siliconed containers for 18–24 hr at 4° C.

As has been shown before (Margolis, 1958*a*), there is an excess of component A relative to component B in normal plasma. The amount of activated component A present in the glass-contacted sample is thus sufficient to 'consume' not only its own component B but also that of the intact plasma with which it has been mixed.

(2) *Plasma depleted of components A and B* (*A+B-dep plasma*). Intact plasma was rotated with 1 g of ballotini/ml. for 60 min and then removed and kept in siliconed containers as above. It was used on the next day or kept frozen at −20° C for up to 1 week.

After glass treatment some specimens were heated to 56 or 61° C for 20 min. The precipitated fibrinogen was discarded after centrifuging. *It should be stressed that A + B-dep plasma still contains most of the original HF*, whose complete removal requires the use of much larger surface areas (about 5000 cm²/ml.), as is shown in Fig. 4.

(3) *Alkaline eluate from glass.* Glass ballotini which were bathed for 2–5 min in plasma or derivatives (1 g ballotini/ml. plasma) were washed with 10 changes of 0·9 % NaCl. They were next suspended in a volume of saline ¼ that of the original plasma, and sufficient 0·1 N-NaOH was added to raise the pH to 10–11. The beads were then agitated for 2–3 min and the supernatant was transferred into siliconed tubes. After addition of a little 5 % $NaHCO_3$ (0·02 ml./ml. of eluate) the eluate was brought back to neutrality with 0·1 N-HCl. Eluates from kaolin (50 mg light kaolin/ml. of plasma) were prepared as above but with the difference that the supernatant was repeatedly centrifuged before neutralizing, in order to remove the last traces of kaolin. When unheated plasma was used for 'coating' kaolin, a fine precipitate formed in the eluate below pH 8·0. This was spun down and discarded. Plasma heated to 56° C yielded eluate which remained clear in neutral solution.

(4) *Indirect test for activation of plasma by glass and biological assays of the formed plasma kinin on rat uterus.* The test has been called indirect (Margolis, 1958*a*) because it is not the amount of kinin released within the test sample itself which is measured (as in the direct method), but the ability of the test sample to liberate the kinin from intact 'substrate' plasma. The test is thus carried out in two stages.

Samples of plasma, etc., were rotated in glass tubes with 1 g of glass ballotini/ml. for 2–3 min. As soon as the beads had settled (within 10–15 sec) 0·1 ml. of the supernatant was added to 0·2 ml. of intact substrate plasma in siliconed tubes. After 2 min incubation, 0·1 ml. of the mixture was applied to one horn of a rat uterus suspended in oxygenated Ringer's solution (Armstrong *et al.* 1957) in a 5 ml. siliconed organ bath kept at 30° C. The uteruses were from virgin albino rats (180–200 g) injected with 50 μg of stilboesterol 24–48 hr previously. The latent period of contraction was measured with a stopwatch. The recording lever was counterbalanced with a cylindrical float suspended in a beaker of water; thus the weight lifted was proportional to the height of the contraction. This arrangement gave more reproducible records than with a fixed counterbalance.

With the exception of the experiments in Fig. 3 the test samples were usually diluted with 2 parts of 0·9 % NaCl immediately before activation. This was introduced for convenience and economy only. Qualitatively identical results were obtained with undiluted samples (cf. Fig. 3). In either case the intact substrate plasma was always used undiluted.

The results illustrated below are representative of a large number of experiments. In most figures the individual panels in the records (e.g. *a*, *b*, *c*) show tests carried out on different days; the contractions are therefore strictly comparable only within each panel.

RESULTS

The dosage of the test samples was such as to give a strong contraction with fully activated plasma, after a latent period of 5–10 sec, and no response within 1 min with an equal dose of intact plasma. Figure 2 illustrates that with indirectly activated samples a full-scale response of the uterus can usually be said to represent activity at least 50 (never less than 20) times greater than in samples eliciting no response. The present conclusions are based on the difference between these two extremes and no further quantitative treatment was attempted.

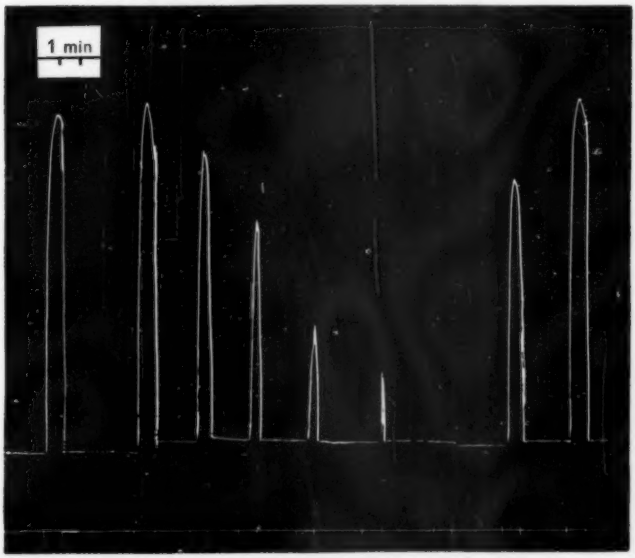

Fig. 2. Rat uterus contractions, as in all subsequent figures. Dose–response pattern of the kinin released from intact plasma by the addition of varying dilutions of glass-activated test plasma (see Methods 4). Component B-depleted plasma, diluted with 2 parts of 0·9 % NaCl, was rotated for 3 min with 1 g of glass ballotini/ml. The supernatant was diluted with 0·9 % NaCl; 0·1 ml. of this final dilution (1 to 1/80 as shown below tracing) was then added to 0·2 ml. of intact substrate plasma in siliconed tubes, incubated 2 min, and 0·1 ml. of this mixture was applied to the uterus for 1 min. L.P. = latent period of each contraction.

The interdependence of HF and Component A

Although neither HF-deficient (HF-dep) nor A + B-deficient (A + B-dep) plasma can be activated separately by contact with glass, a mixture of the two (Fig. 3, tests 5 and 7) behaves like normal plasma (test 4). HF and component A cannot therefore be identical but must be complementary to each other. As is shown in Fig. 3 the mutual supplementation takes place only when the two plasmas are mixed first and then treated with glass (tests 5 and 7) but not when they are exposed to glass surfaces separately and mixed immediately thereafter (test 6).

Evidently glass, HF and component A form the minimal reactive system in the first stage of the kinin-releasing mechanism. The reason for this will become apparent when the findings shown in Fig. 4 have been considered.

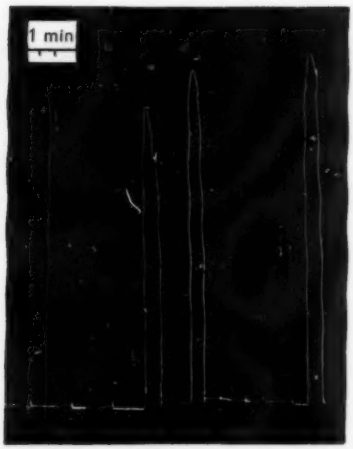

Fig. 3. Ability of HF and component A to supplement each other in kinin formation. Undiluted plasma test samples, enumerated below, were rotated for 2 min with 1 g of ballotini/ml. 0·1 ml. of supernatant was then incubated with 0·2 ml. of intact normal plasma in siliconed tubes for 2 min and 0·1 ml. of this mixture was tested on the rat uterus.

Test samples: 1, HF-dep (Hf-deficient) plasma. 2, A + B-dep (component A and B-depleted) plasma. 3, Control mixture of equal volumes of HF-dep and A + B-dep plasma, incubated without glass ballotini. 4, Normal plasma. 5 and 7, Mixture as in test 3 but rotated with ballotini. 6, HF-dep and A + B-dep plasma rotated with ballotini separately and mixed after removal from glass.

The fixation of HF to the glass surface

Glass previously 'coated' with normal plasma, or derivatives containing HF, fully activates HF-dep plasma. This property persists after repeated washing of the glass with water or neutral saline solution and, after one coating, the same glass can be used for the activation of several successive samples of HF-dep plasma without any apparent loss of activity. In Fig. 4*a* (tests 3–5) this phenomenon is illustrated by using A + B-dep plasma, which contains HF, as the coating reagent. Figure 4*b* shows that glass coated with preparations deficient in HF, such as natural HF-dep plasma or plasma deprived of HF by heating above 60° C (test 4) or by adsorption with more than 20 mg kaolin/ml. (tests 2 and 3), does not activate HF-dep plasma. There is also no evidence of activation when the order of the procedure in Fig. 4*a* is reversed, that is when the glass beads are pre-treated with HF-dep plasma and then rotated with A + B-dep plasma (Fig. 4*c*, test 4).

Together with the data in Fig. 3, these results suggest the following

mechanism: (*a*) HF is activated by adsorption to the glass surface and remains active only in the adsorbed state; and (*b*) component A is activated only after the adsorption of HF on to the glass. It should now be clear why the mixture of HF-dep and A + B-dep plasmas contacted in separate tubes (Fig. 3, test 6) was inactive. If all the activated HF in the A + B-dep plasma is discarded with the beads it obviously cannot react with the component A in the HF-dep plasma in the other tube.

The experiments with kaolin (tests 1, 2, 3 in Fig. 4*b*) show that in order to remove all the HF from normal plasma, a surface area of about 5000 cm^2/ml. is required, the specific surface area of light kaolin being of the order of 10 m^2/g.

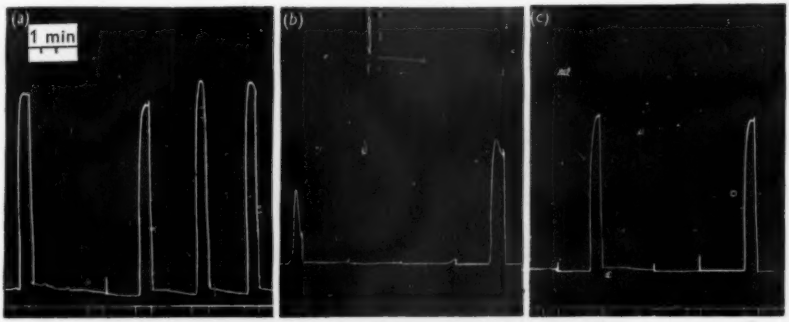

Fig. 4. Fixation of HF on to glass surface. Glass ballotini were coated as indicated below and then washed with 10 changes of 0·9 % NaCl (see Methods).

(*a*) *Test samples*: HF-dep plasma. *Treatment of ballotini* (washed with further 10 changes of saline before tests 3–5): 1, A + B-dep plasma, the source of HF. 2, 0·9 % NaCl. 3, Ballotini already used in test 1. 4, Ballotini from test 3. 5, Ballotini from test 4.

(*b*) *Test samples*: HF-dep plasma. *Ballotini* coated with: 1–3 Normal plasma adsorbed with kaolin for 8 min: (1, 20 mg/ml. 2, 50 mg/ml. 3, 100 mg/ml). 4, Normal plasma heated to 61° C for 20 min. 5, Normal plasma.

(*c*) *Test samples*: 1, 2, 5, HF-dep plasma. 3, 4, A + B-dep plasma. *Treatment of ballotini*: 1, 0·9 % NaCl. 2, A + B-dep plasma. 3, 0·9 % NaCl. 4, HF-dep plasma. 5, From test 2.

The failure of glass to remove components A and B from HF-dep plasma

The evidence for the reaction sequence postulated above was strengthened by experiments in which the conditions were so arranged that HF-dep plasma was found to be more effective than normal plasma in these activation tests. These experiments were based on the observation that

in the process of activation components A and B are consumed (Margolis, 1958*a*). Since in the absence of HF none of the later stages can take place (cf. Fig. 1), we can anticipate that after treatment with glass these components would not disappear from HF-dep as from normal plasma.

This hypothesis was subjected to the tests shown in Fig. 5 in which HF-dep plasma was treated with glass, which would normally have exhausted components A and B, and could then still be used as a source of these components in the test system. The results of Fig. 5*a* show that HF-dep plasma is not depleted of component B by a direct treatment with ballotini (see tests 1 and 2) which is more than sufficient to remove this component from normal plasma (test 3). Figure 5*b* shows that component B

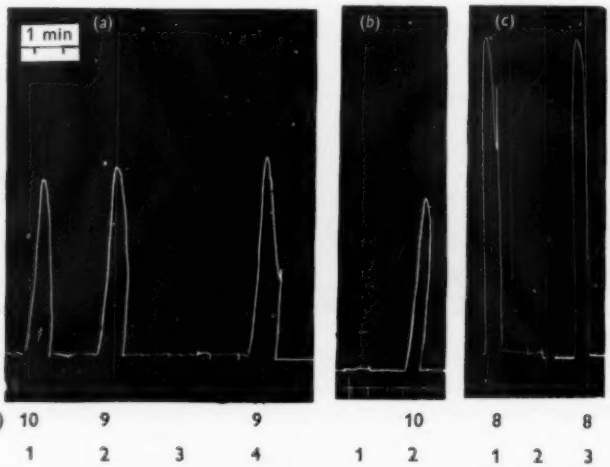

Fig. 5. Persistence of components A and B in HF-dep plasma after treatment with glass ballotini. In (*a*) and (*b*) tests for depletion of component B, in (*c*) test for depletion of component A; see text.

(*a*) B-dep plasma was activated with ballotini as before and added to: 1, HF-dep plasma. 2, HF-dep plasma rotated 5 min with 1 g ballotini/ml. and then incubated at 4° C in siliconed tubes for 18 hr; this would completely remove component B from a normal plasma which then fails to release kinin when used as substrate, as is shown in 3, Normal plasma treated as in 2. 4, Normal intact plasma.

(*b*) B-depleted activated 'test' plasma as in (*a*). Substrate 1, Normal plasma activated 2 min with 1 g ballotini/ml. and added to an equal vol. of HF-dep plasma; mixture incubated at 4° C for 18 hr in siliconed tubes (see Methods: 'B-dep plasma'). 2, As in 1 but the plasmas were incubated 2 hr in separate tubes before mixing.

(*c*) Normal plasma substrate. Ballotini coated with A + B-dep plasma (source of HF). Test plasmas: in 1, normal; in 2, A + B-dep (obtained from normal plasma by rotation for 1½ hr with equal wt. of ballotini and incubation at 4° C for 18 hr); in 3, HF-dep treated as in 2.

can be removed from HF-dep plasma (test 1) by the addition of glass-treated normal plasma which contains an excess of activated component A (see Methods). Since the latter decays on incubation, component B was found to persist in a control sample (test 2) in which the two plasmas were mixed as in test 1 but after a preliminary 2 hr incubation in separate siliconed tubes.

Figure 5*c* shows that HF-dep plasma can still serve as an efficient source of component A after prolonged rotation with glass ballotini (test 3). In these experiments HF had to be supplied to complete the reacting system (cf. Fig. 1). This was done by coating the beads used in the final tests (not those employed for the prolonged rotation) with HF-containing A + B-dep plasma (cf. Fig. 4*a*). Without this step, component A in HF-dep plasma could not be activated and would therefore remain undetectable. Normal plasma treated and tested in the same manner was quite inactive (Fig. 5, test 2).

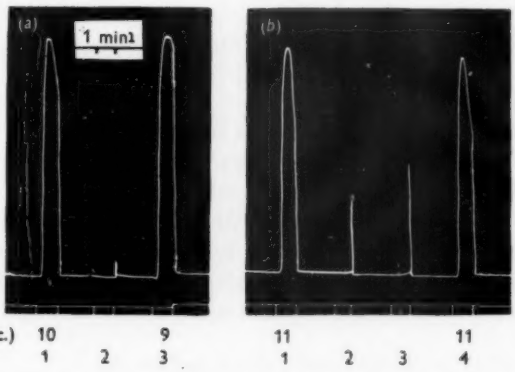

Fig. 6. Elution of HF from glass at alkaline pH. Explanation in text. *Test samples*: test 3 in *a*, normal plasma; remaining tests in *a* and *b*, HF-dep plasma. *Ballotini* were coated with the preparations indicated below and washed 10 times with 0·9 % NaCl solution before each test. In *a*, tests 2 and 3, the 4th–7th washings were at pH 10·0; in the remaining tests the pH of the saline was 7·0 throughout. *Coating of ballotini*: *a*, in all tests AB-dep plasma. *b*, Neutralized alkaline eluate (see Methods 3) from glass treated with: 1, normal plasma, 2, HF-dep plasma; 3, normal plasma heated to 60° C for 20 min; 4, A + B-dep plasma (which contains HF).

Elution of the adsorbed HF from glass

When glass, coated with preparations containing HF, is washed at pH 10–11 it loses its ability to activate HF-dep plasma (Fig. 6*a*, test 2), although it will still activate normal plasma (Fig. 6*a*, test 3). This is presumably due to the removal of the adsorbed HF at alkaline pH (cf. Bangham, Pethica & Seaman, 1958). The HF activity in the eluate can be

recovered by re-adsorption on to fresh glass beads after neutralizing the solution with dilute HCl (Fig. 6*b*, tests 1, 4).

Eluates prepared from beads coated with preparations lacking in HF are inactive (Fig. 6*b*, tests 2, 3), which shows that the effect is not an artifact incidental to the elution procedures but is specifically dependent on the presence of HF.

Other activation products on the glass surface

So long as the material on the glass surface is derived from plasma previously exhausted of components A and B, the effect of such glass can be attributed to the presence of HF alone. When, however, the beads are coated with normal plasma still containing component A, they acquire additional properties. Thus, apart from activating HF-dep plasma they will also activate A + B-dep plasma. The activation of A + B-dep plasma is not nearly as complete as that of HF-dep plasma, but it cannot be dismissed as a trace effect because here also successive samples of the A + B-dep plasma can be activated with the same coated beads. Since A + B-dep plasma is not grossly deficient in HF, the correction in this case cannot be explained only by the presence of HF on the beads. It must, therefore, be due to the adsorption of further activation products on the surface. Even so, this is still dependent on the presence of HF because, as is shown in Fig. 4*c*, test 4, glass treated with HF-dep plasma does not activate A + B-dep plasma. The above observations are relevant to the present problem, inasmuch as these secondary adsorbed agents may be present as active impurities and may give rise to difficulties in experiments in which elution is used for the purification of HF (see below).

Properties of the eluted material

The results illustrated in Fig. 6 have shown that the eluted material is active when reapplied to fresh glass. It is of theoretical interest to establish whether it also remains active in solution. In attempting to answer this question it soon became apparent that a negative result would be much more significant than a positive result. For if a given preparation is inactive, we may conclude that it contains no activated HF, but the converse statement need not be true because activity in solution may conceivably be due to further products of the reaction. The observations below must therefore be interpreted accordingly.

Eluates prepared from glass treated with A + B-dep plasma which contains HF but is devoid of the remaining known components of the system were inactive in siliconed tubes (Fig. 7*a*, test 1). Further, when such an eluate, glass and HF-dep plasma were brought together in various combinations (Fig. 7*a*, tests 2–7) activation could only be detected when

the eluate came in contact with glass at the same time as the HF-dep plasma (tests 2, 7) or before the addition of the latter (test 5). These observations suggest that HF eluted from the glass surface does not remain active in solution but returns to its precursor form, or at least that it does so as soon as it is added to plasma (normal or HF-dep).

On the other hand eluates prepared from glass treated with B-dep or with whole normal plasma (which contain respectively component A and components A and B in addition to HF) released the kinin when added in siliconed tubes to intact substrate plasma even in the absence of glass beads (Fig. 7*b*, test 2). It seems reasonable to suppose that this activity

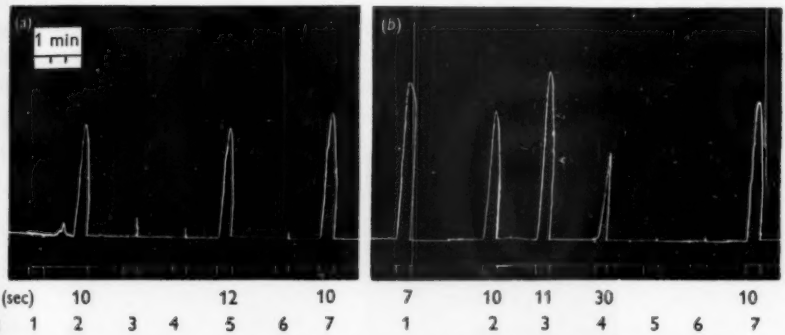

Fig. 7. Properties of eluted HF (see Text). Alkaline eluates were prepared from ballotini coated either with A+B-dep (in *a*) or with B-dep plasma (in *b*). The test samples were mixtures of equal volumes of 0·9 % NaCl, eluate and HF-dep plasma in various combinations. They were either rotated with glass ballotini (G) or incubated in siliconed tubes (S) for 3 min and then added to intact substrate plasma for tests on uterus.

(*a*) *A+B-dep eluate.* Test samples: 1, Eluate (S). 2, Saline, eluate, HF-dep plasma (G). 3, Eluate, HF-dep plasma (S). 4, Saline, eluate (G). 5, Saline, HF-dep plasma (G); ballotini from test 4. 6, Saline, HF-dep plasma (G). 7, Eluate, HF-dep plasma (G).

(*b*) *B-dep eluate.* Test samples: 1, B-dep plasma (G). 2, Eluate (S). 3, Saline, eluate, HF-dep plasma (G). 4, Saline, eluate, HF-dep plasma (S). 5, Saline, eluate (G). 6, Saline, HF-dep plasma (G). 7, Saline, HF-dep plasma (G); ballotini from test 5.

is not due to HF being active in solution but to the presence in the eluate of a further activation product or products (e.g. activated component A), which, unlike HF, does not revert to the precursor form after removal from the glass surface. Otherwise the results in this panel are similar to those in Fig. 7*a*, except that when the mixture of the eluate, saline and HF-dep plasma was incubated without contact with glass some residual activity could still be detected (test 4).

More concentrated preparations of the eluted proteins than the one

used in Fig. 7*a* were obtained by using kaolin as the adsorbent. These were, however, already active before the exposure to glass. It is not yet clear whether this discrepancy between the eluates from glass and those from kaolin is due to quantitative factors, or is of a more fundamental nature. Theoretical considerations favour the latter alternative (see Discussion).

Failure of glass pre-treated with HF-dep plasma to adsorb HF from normal plasma

Glass which had been treated with HF-dep plasma loses much of its ability to accelerate coagulation of normal plasma (Hardisty & Margolis, 1959). We interpreted this as being due to inert substances which combine with glass and block its capacity to adsorb HF. The same phenomenon can be demonstrated in the activation of the kinin-releasing mechanism. Thus, there was no detectable activation in a mixture of normal and HF-dep plasma when the HF-dep plasma was added to the glass beads first and

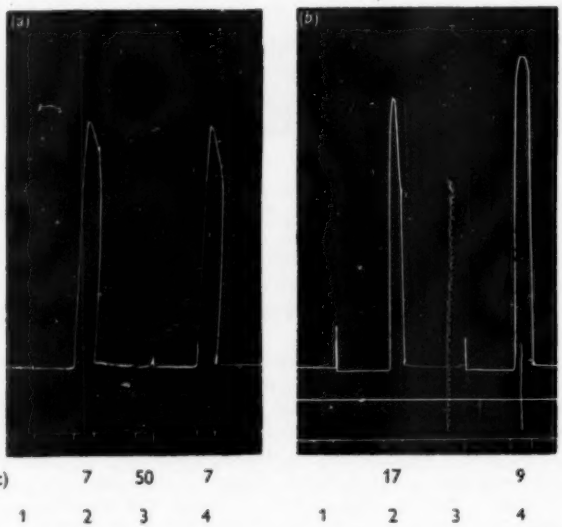

Fig. 8. Suppression by HF-dep plasma (*a*) and by rat kidney homogenate (*b*) of glass activation of normal plasma. See text.

(*a*) To 0·4 g of ballotini were added 0·2 ml. of normal diluted plasma (1/10 in 0·9 % NaCl) and 0·2 ml. of undiluted HF-dep plasma. The tubes were rotated for 3 min and the supernatant tested. *Test samples*: 1, HF-dep plasma alone. 2, Normal plasma added to ballotini 30 sec before HF-dep plasma. 3, HF-dep added to ballotini 30 sec before normal plasma. 4, Normal and HF-dep plasma mixed in a siliconed tube and added to ballotini together.

(*b*) Indirect activation test on normal plasma using ballotini treated with homogenized rat kidney (see Text) and then washed 10 times with neutral 0·9 % NaCl. *Concentration of homogenate*: 1, Undiluted. 2, 1/10. 3, 1/2. 4, 0 (control).

was then followed by normal plasma (Fig. 8*a*, test 3). On the other hand, full responses were obtained when the order of addition was reversed (test 2), or when the two plasmas were first mixed in a siliconed tube and then exposed to glass (test 4). This deleterious effect of HF-dep plasma could not be removed by washing the beads with saline, which is a clear indication that the surface had become blocked. There is, however, nothing in these results to point to a specific inhibitor in HF-dep plasma. Similar observations were made with normal plasma in which HF was destroyed by heating to 61° C, as well as with some tissue extracts when a number of tissues were tested for the presence of HF. In Fig. 8*b* the material used was rat kidney, previously perfused with saline to remove all traces of blood, since rat plasma contains HF. Instead of HF, a powerful blocking effect was discovered.

The most obvious interpretation of the above results is that there are in plasma and tissues substances which compete with HF for the adsorptive sites on the glass surface.

DISCUSSION

HF is actually deposited on the glass surface

In the preceding description the relevant adsorbed material was referred to as HF. However, in the absence of a positive chemical identification, it must be shown that the substance in question: (*a*), can replace HF in HF-dep plasma; (*b*), cannot be obtained from HF-dep plasma and (*c*), corrects only HF deficiency.

The criteria (*a*) and (*b*) are easily satisfied. But the specificity of the activity (*c*) can only be assessed by exclusion. All that can be said at present is that it is possible to obtain preparations which correct HF deficiency but have no such effect on plasma containing HF and deficient in other hitherto recognized components of the system. Such negative evidence can, of course, never be entirely satisfactory. Additional proof, however, may be implicit in the experiments of Fig. 7.

Thus, while an already active substance (whether on glass or in solution) could be either HF or a further product of the reaction, it is probable that a preparation which is not active to begin with, but becomes so in contact with glass, must contain HF. If this reasoning is admissible, then it follows that HF must have been adsorbed to the glass from which the eluates such as those in Fig. 7*a* were obtained.

Activity of HF probably only in the adsorbed form

According to previous concepts (Margolis, 1958*a*; Soulier *et al.* 1958) the activated HF was supposed to be released back into the plasma where it could be recognized by various tests. The present observations speak

against such an interpretation. First, while A+B-dep plasma seems to contain an ample supply of HF, this plasma when shaken with glass and then immediately added to intact plasma does not release any kinin (Fig. 3), which would be expected should any activated HF have escaped from the glass surface. Secondly, there are the observations that one sample of 'coated' glass beads completely corrects the defect in successive specimens of HF-dep plasma (Fig. 4). This indicates that there is no apparent loss of the adsorbed HF from the surface and also that the amount of HF combined with the surface is adequate to account for all the detectable activity. Thus we can probably accept without further reservations that only this part of HF which is actually carried on the surface is capable of initiating further events in the plasma.

The above reasoning applies to the state of HF as it occurs in the plasma. It does not necessarily apply to HF eluted from glass by treatment with alkali. It would, however, be difficult to explain the results of Fig. 7*a* except by assuming that the surface activation of HF is, at least partly, reversible. This agrees with present concepts of protein denaturation (cf. Fraser, 1957). Activation of HF by adsorption most likely involves some unfolding of the protein molecules which results in uncovering of enzymically active groups. The degree of unfolding (i.e. of denaturation) depends on the amount of available surface. With a relatively small surface such as is provided by glass beads, this is restricted by 'overcrowding' and is evidently insufficient to produce irreversible changes in the (tertiary) protein configuration. Hence, upon elution, HF tends to resume its native precursor form. The presence of a larger surface area (e.g. kaolin) leads to a more advanced denaturation due to the disruption of stronger intramolecular bonds. The protein structure is still sufficiently specific to retain its enzymic activity but it cannot return to its native form. Thus HF eluted from kaolin remains active in solution. Finally, when the surface is so large that it is no longer a limiting factor, not only the tertiary but also the secondary convolutions of the adsorbed proteins are disrupted and all specific function is lost. Under these conditions HF activity can no longer be detected (unpublished observations).

Some inadequacies in the present scheme

The mode of action of HF in kinin release seems to be fairly intelligible and is entirely consistent with the studies of its role in coagulation (Hardisty & Margolis, 1959) and the formation of a permeability factor (Margolis, 1959). However, there still remains a residue of facts which cannot be easily fitted into the scheme outlined in Fig. 1. These refer to the nature of component A, which was originally defined as the immediate precursor of the kinin-releasing agent detected by the indirect activation

test (Margolis, 1958*a*). Since in the indirect test glass is removed from the system, the activated component A must be present in solution. Yet, as was stated above, some of the lost component-A activity in A + B-dep plasma can be partially replaced by substances other than HF, which are adsorbed to the glass particles. This makes it difficult to account for the functions of component A in terms of a single factor. It may thus be more proper to speak of a 'complex A' rather than 'component A'. The semantic difficulties could, of course, be avoided if the experiments could be described without any reference to incompletely specified factors but such an account would then be extremely cumbersome. The terms 'component A' and 'B' were therefore retained for the present and, with the reservations mentioned, they are still useful in providing a relatively simple theoretical framework on which to hang a large number of diverse observations.

SUMMARY

1. The first detectable stage in the release of plasma kinin by contact with glass is the adsorption of Hageman factor (HF) by the glass surface.

2. The adsorbed HF activates catalytically a 'component A' which then interacts with a 'component B' in the plasma.

3. At the pH of plasma the activated HF remains fixed to the surface, but it can be eluted with alkali and then reapplied to a fresh surface after neutralization. There is some evidence that the eluted HF returns to its precursor form in solution.

4. In the presence of HF a further activation product is adsorbed by the glass surface. This is distinct from HF and remains active in solution upon elution.

5. Plasma and tissues contain substances which block the capacity of glass to adsorb HF.

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THE EFFECT OF DEOXYCORTICOSTERONE ON SALT AND SUCROSE TASTE PREFERENCE THRESHOLDS AND DRINKING BEHAVIOUR IN RATS

BY A. HERXHEIMER* AND D. M. WOODBURY

From the Department of Pharmacology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.

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In an experiment in man, de Wardener & Herxheimer (1957) reported a lowering of the salt taste threshold during a period of high water intake. Other observations made in the experiment suggested that sodium was transferred from bone and/or cells into the extracellular fluid, and it seemed possible that the lowering of the taste threshold might be related to a loss of sodium from cells, either in the taste receptors or in the brain. It was therefore of interest to examine in an animal the effect on taste thresholds of a procedure known to lower the intracellular sodium concentration in the brain. The chronic administration of deoxycorticosterone acetate (DOCA) to rats is such a procedure (D. M. Woodbury, unpublished observations). In the present experiment salt and sucrose taste preference thresholds have been determined in rats before and during treatment with DOCA.

METHODS

The subjects were twelve male albino rats, aged approximately 90 days at the beginning of the experiment (December 8, 1958). They were individually housed in a constant-temperature room (25–25·5° C) with 24 hr illumination. The food consisted of powdered Purina Rat Chow (0·5 % NaCl). From December 8 to January 4 each rat received 10 g of this food daily; from January 5 until the end of the experiment 13 g was given daily, because there had been some loss of weight. The food offered was always completely consumed. The amount of food was restricted in order to keep the daily intake constant.

The animals were allotted at random to two groups; in one group salt and in the other sucrose taste thresholds were determined. The standard two-tube procedure was used, one tube containing the test solution, the other distilled water. Each test concentration was offered for 48 hr, the position of the solution being varied so that it occupied each side of the cage for 24 hr. The test solutions were presented in descending order of concentration except for a preliminary ascending series (November 17–December 5) which served to introduce the animals to the experimental situation. Each rat had the same two drinking tubes throughout each test series. At each change of concentration the tubes were alternated, so that the tube that had contained the solution for the previous 48 hr was filled

* Present address: Department of Pharmacology, London Hospital Medical College, London, E. 1.

with water, and vice versa. The test solutions which were used are given in the legends to Figs. 1–2.

The taste preference threshold was taken to be the lowest concentration at which more test solution was drunk than water, provided that the difference exceeded $\frac{1}{3}$ of the total fluid intake for the 48 hr. This threshold is marked by an arrow in each of the curves in Figs. 1 and 2; below the threshold an animal drinks water and test solution in approximately equal amounts. In 10 out of 30 threshold determinations irregularities of intake introduced some difficulty, as in some examples in Fig. 2. Then intakes at two successive concentrations (i.e. during 4 days) were added, and if the intake of test solution exceeded in the 4-day period the intake of water it was assumed that the threshold had not yet been passed. An excess greater than $\frac{1}{3}$ of the total intake was arbitrarily regarded as significant.

In the first test series (December 11–January 6) taste thresholds were determined for all the rats. On January 7 three rats in each group of six received an implant of seven 15 mg DOCA pellets under the skin of the back, the pellets being deposited singly at different sites. A 'sham' operation was performed on the other three rats in each group. Five days later the second series of taste threshold determinations was begun. Finally, on February 18, DOCA pellets were implanted in the animals that had acted as controls in the second test series; their taste thresholds were then determined for a third time (February 22– March 15). Between test series water was presented in both tubes.

RESULTS

The results are given in Table 1. The salt preference threshold fell sharply in each of the DOCA-treated animals (Fig. 1); little change occurred after sham treatment in the controls. The sucrose preference thresholds were more variable than those for salt and showed no consistent change, either in the DOCA-treated animals (Fig. 2) or the sham-treated controls. In the second part of the experiment the six animals that were previously controls were given DOCA. As before, the salt preference threshold fell during DOCA treatment, while the sucrose preference thresholds behaved inconsistently.

In addition to the changes in preference threshold certain changes occurred in the total fluid intake (Figs. 1 and 2). Invariably the intake was highest when the most concentrated solution of a series was presented, and then declined as the concentration was lowered, until it reached a 'basal' level. This basal intake remained essentially unchanged when the sapid solution was withdrawn and replaced by water at the end of the test series. The strength of the preference for a test solution can be assessed by the type of drinking behaviour. Below the preference threshold similar amounts of test solution and water are taken. At and above the preference threshold an animal drinks more test solution than water, but the total fluid intake remains the same. Then at higher concentrations it begins to drink so much more of the test solution that the total fluid intake increases, indicating that the preference is stronger than before. On the average the total intake exceeded the basal values when sodium chloride at a concentration about 20 times the salt preference threshold was offered or a sucrose solution about 6 times threshold. These ratios were not affected by DOCA.

Individuals differed widely in basal intake. The total fluid intakes in the first test series, before DOCA treatment, were similar in the control and treatment groups. In the second test series both the sham-operated and the DOCA-treated rats tended to drink more than in the first series, but the basal intake of the treated animals was much higher than that of the controls.

TABLE 1. Taste preference thresholds

Test period	Rat no.	Thresholds for NaCl (g/100 ml.) 1 Before sham implantation	2 After sham implantation	3 After DOCA implantation
Control animals: effect of sham implantation, followed by DOCA implantation	7	0·005	0·009	0·003
	9	0·009	0·016	0·0016
	11	0·009	0·009	0·0005
Geometric mean		0·007	0·011	0·0013
		Before DOCA implantation	After DOCA implantation	
Treated animals: effect of DOCA implantation only	8	0·03	0·00005	
	10	0·009	0·003	
	12	0·05	0·0009	
Geometric mean		0·024	0·0005	

Test period	Rat no.	Thresholds for sucrose (g/100 ml.) 1 Before sham implantation	2 After sham implantation	3 After DOCA implantation
Control animals: effect of sham implantation, followed by DOCA implantation	1	0·25	0·4	0·6
	3	0·1	0·4	0·1
	5	0·6	0·025	0·15
Geometric mean		0·25	0·16	0·2
		Before DOCA implantation	After DOCA implantation	
Treated animals: effect of DOCA implantation only	2	0·04	0·15	
	4	0·15	0·04	
	6	0·15	0·015	
Geometric mean		0·10	0·04	

Other observations

The manner in which the solutions were presented to the animals was designed to eliminate interference from any preference that a rat might have for one of the two drinking positions or for one of the tubes. Eight of the rats developed more or less strong position preferences; in some animals gradual changes in position preference occurred. Tube preference was noted only occasionally and was slight.

The amount of DOCA absorbed by the six animals that received implants was estimated by removing most of the pellets at the end of the second test series and weighing them. The mean weight per pellet removed

after 42 days was 7·5 mg (S.D. 1·0); the animals therefore absorbed DOCA at the mean rate of 1·2 mg/day. Since their mean weight during the period of DOCA treatment was 297 g, the average daily dose was 4·0 mg/kg.

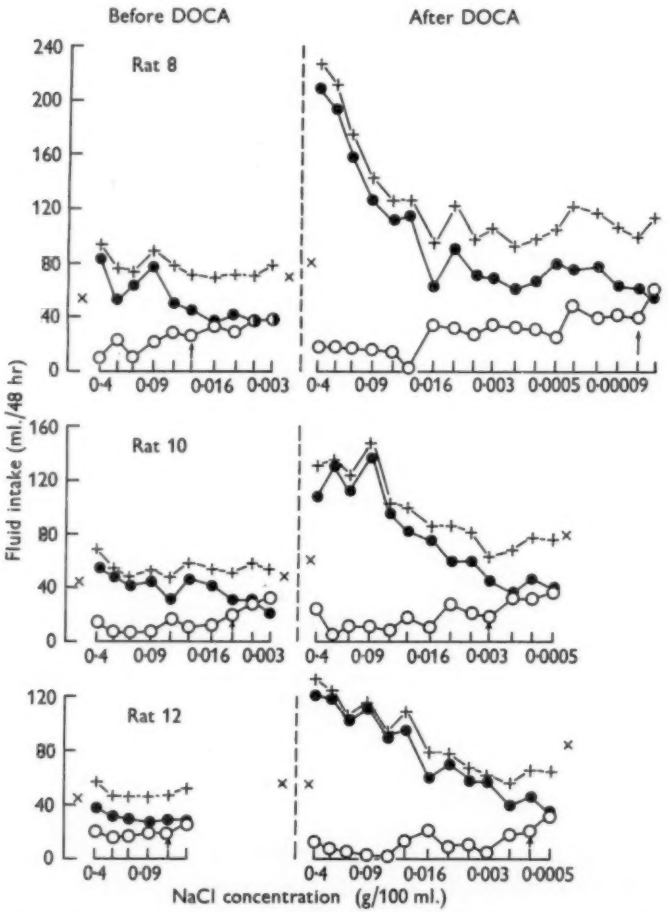

Fig. 1. Total fluid (+), salt solution (●) and distilled water (○) intakes of rats 8, 10 and 12 before and after implantation of DOCA pellets. Concentrations of sodium chloride (g/100 ml.) of 0·4, 0·25, 0·16, 0·09, 0·05, 0·03, 0·016, 0·009, 0·005, 0·003, 0·0016, 0·0009, 0·0005, 0·0003, 0·00016, 0·00009, 0·00005, 0·00003 were presented in successive periods of 48 hr; the second test series began 5 days after the implantation of DOCA. The lowest concentration of salt at which a rat drank more salt solution than water is the salt preference threshold(↑). The total intake for days on which water was presented in both drinking tubes is shown separately (×). Usually, two such days preceded and two followed each test series.

DISCUSSION

The changes in preference threshold following DOCA treatment were much greater and much more consistent for salt than for sucrose. It is unlikely that the changes in the sucrose threshold were related to the treatment. A lowered taste threshold for NaCl has also been observed in

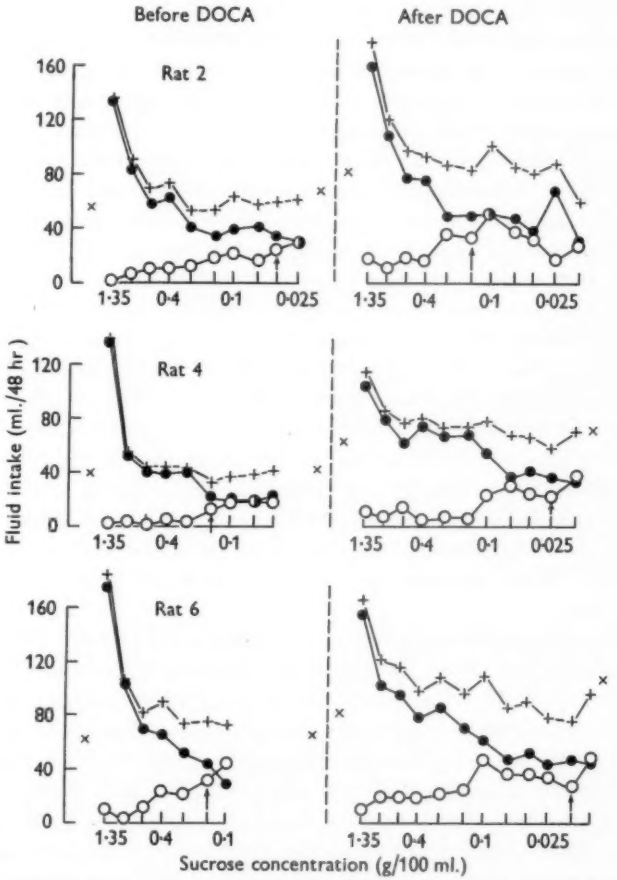

Fig. 2. Total fluid (+), sucrose solution (●) and distilled water (○) intakes of rats 2, 4 and 6 before and after DOCA implantation. Concentrations of sucrose (g/100 ml.) of 1·35, 0·9, 0·6, 0·4, 0·25, 0·15, 0·1, 0·06, 0·04, 0·025, 0·015, 0·01 were presented in successive periods of 48 hr; the second test series began 5 days after the implantation of DOCA. The sucrose preference threshold is indicated (↑). The total intake for days on which water was presented in both drinking tubes is shown separately (×). Usually two such days preceded and two followed each test series.

man during a period of high water intake (de Wardener & Herxheimer, 1957) and during sodium deprivation (Yensen, 1958*a*). It may be supposed that a decrease in the intracellular sodium concentration occurred in both these experiments. In a later experiment Yensen (1958*b*) studied the effect of water deprivation, which would be expected to cause a rise in intracellular Na concentration, and found that this led to a rise in the salt taste threshold.

The findings also agree with the observation that DOCA increases the appetite of rats for salt (Rice & Richter, 1943; Braun-Menéndez & Brandt, 1952). It is difficult, however, to relate the present results to Richter's (1939) and Bare's (1949) finding that the salt preference threshold is lowered by adrenalectomy. Both adrenalectomy and treatment with DOCA increase the salt appetite and lower the preference threshold, yet when adrenalectomized rats are given DOCA their salt consumption returns to normal (Richter, 1941). Adrenalectomy and DOCA treatment have opposite effects on the electrolyte distribution in the rat cerebral cortex, and on the electroshock seizure threshold (Woodbury, Timiras & Vernadakis, 1957). The observation that DOCA-treated rats become supersensitive to insulin and to anaphylaxis has led to the suggestion that DOCA may inhibit glucocorticoid secretion (Rosenberg, Woodbury & Sayers, 1952). If DOCA in fact leads to glucocorticoid deficiency, as does adrenalectomy, this might mediate the fall in salt preference threshold; the effect of cortisone on the threshold will have to be investigated. It will also be necessary to determine whether the effects of adrenalectomy and of DOCA treatment on the electrolyte distribution in other parts of the brain, particularly the hypothalamus, differ from those seen in the cortex (Stellar, 1954).

The results reported here do not help to locate the effect of DOCA on the salt preference threshold; it could be produced either at the sensory receptor, or centrally. However, the fact that adrenalectomy produces a similar effect may offer a clue to the site of action of DOCA, for it has been found that adrenalectomy affects neither the taste receptor threshold for salt (Pfaffmann & Bare, 1950) nor the psychophysical threshold, determined by a forced discrimination technique (Carr, 1952; Harriman & MacLeod, 1953). The first observation indicates that the lowering of the salt preference threshold after adrenalectomy is not due to a fall in receptor threshold; the second suggests that the central mechanism involved in psychophysical threshold determinations is separate from that mediating spontaneous taste preference behaviour. It may be that DOCA treatment, like adrenalectomy, affects only the latter mechanism.

Hitherto little attention has been paid to the phenomenon of extra fluid intake in taste preference experiments, although its existence is

apparent in previous work (Richter, 1939; Bare, 1949; Young, 1949; Fregly, 1956). The extra fluid intake cannot be attributed to the need for increased water to excrete the extra solute load, because in the experiments with salt, where this argument might apply, the rats drank far more than the amount they required for this purpose. Moreover, the rats given sucrose, which is completely metabolized, showed a similar extra fluid intake. An indication that the taste of the solution may be responsible comes from the work of Sheffield & Roby (1950), who found that a solution of saccharine was an effective reward for learning in the rat. The present results give further support to the conclusion of Weiner & Stellar (1951) that 'the rat is so built that its rate of drinking is directly determined by the stimulus intensity of the. . . solution'. If one ventures to look beyond the austere and cautious wording, this may suggest that the rat drinks these solutions because it likes them, from greed rather than need.

SUMMARY

1. Salt and sucrose taste preference thresholds were determined in rats before and during treatment with DOCA.

2. A fall in the salt preference threshold occurred during DOCA treatment; no fall occurred in the control animals. The sucrose preference threshold did not change consistently in either group.

3. Attention is drawn to changes that occurred in total fluid intake during the preference-threshold determinations. At higher concentrations of salt or sucrose, considerably above the preference threshold, the total fluid intake was progressively greater than at concentrations near the preference threshold.

4. DOCA may exert its effect on the salt taste threshold by altering the intracellular electrolyte distribution in the brain, which it is known to do, or by affecting the taste receptors.

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EFFECTS OF NORADRENALINE AND ADRENALINE ON THE THYROID

BY J. F. MOWBRAY AND W. S. PEART
From the Medical Unit, St Mary's Hospital, London, W. 2

(*Received* 4 *September* 1959)

When Labbé, Tinel & Doumer (1922) first described a patient with a phaeochromocytoma they noticed swelling of the root of the neck in the attacks of sweating, pallor and hypertension. Strombeck & Hedberg (1939) described swelling of the thyroid as at least part of the cause of this increase in neck size.

It has been shown that phaeochromocytomata, in different patients, secrete varying proportions of noradrenaline and adrenaline (von Euler, 1951), and Barnett, Blacket, Depoorter, Sanderson & Wilson (1950) have described swelling of the region of the thyroid in two subjects given infusions of noradrenaline. We have carried out a number of experiments in both man and dogs to study this finding further, with particular reference to the changes in blood flow which occur. A preliminary report of these findings has appeared previously (Mowbray & Peart, 1958).

METHODS

Man

Infusions of noradrenaline and adrenaline were given to fifty-one healthy young adults; forty-nine male and two female. All subjects were at rest on normal diet and infusions were given from 30 to 90 min after their midday meal.

Infusions containing 4 μg/ml. of either noradrenaline or adrenaline in 0·9 % saline were given into an antecubital vein. The infusions were for periods of 30–90 min, and the infusion rate was adjusted so that the systolic blood pressure for most individuals lay between 170 and 190 mm Hg. The dose of both substances required to maintain this lay between 15 and 30 μg/min.

Measurements of blood pressure were made by sphygmomanometer. The measurements of thyroid blood flow were made with a modification of Hensel's heated thermocouple needle (Mowbray, 1959). In this procedure a heated thermocouple is cooled by the tissue blood flow and a reference thermocouple in the same needle compensates for changes in tissue temperature. It was possible to contain the thermocouples and heater in a No. 12 hypodermic needle, which was small enough to be inserted almost painlessly in the thyroid through a small bleb of intradermal procaine solution. Readings of thyroid blood flow were made at 2 min intervals in most subjects, although direct recording of the blood flow has also been used. For this purpose a mirror galvanometer was used for taking spot readings and a DC amplifier with a direct-writing milliammeter for the continuous recordings.

Measurements of the neck circumference were made by sticking a length of tape around the neck and leaving the ends over the front of the neck free. Measurement of the tape plus the gap gave the neck circumference.

Dogs

The dogs used were of a number of breeds weighing about 50–70 lb. (22·7–31·8 kg). Anaesthesia was induced with intravenous sodium thiopentone and was maintained with Nembutal (pentobarbitone; Abbott Laboratories). Laryngeal incubation was carried out, and respiration maintained at a constant rate with a Starling pump. The animals were given heparin 250 u./kg intravenously at the end of the dissection; 100 u./kg was given each subsequent 4 hr.

The thyroids were dissected out and one lobe was used for measurement. There is no isthmus to the thyroid of the dog, and the volume of the isolated lobe was about 1·0 ml. The vessels supplying the lobe were identified and all veins but one were tied; this was cannulated and the effluent blood led through nylon tubing. The drops of blood issuing from the end of this were made to displace a pivoted lever from a reservoir containing saline. The electrical contact between lever and saline was used as input to a Gaddum drop recorder. (The flow was of the order of 30 ml./hr, and it was not necessary to return this to the animal.) A muscular branch of the superior thyroid artery was chosen and cannulated retrogradely so that close intra-arterial infusions of noradrenaline and adrenaline could be given from a constant-rate infusion pump.

Intravenous infusions were given into a femoral vein. Direct recording of blood pressure was made from a mercury manometer attached to a femoral artery cannula.

Measurements of volume change in the thyroid were made by enclosing a lobe in a cylindrical Perspex oncometer and recording with a tambour and writing lever. A change in volume of 0·01 ml., i.e. 1 % of the volume of the lobe, could be easily detected. In order to have confidence in the recordings of volume change it was found necessary to have simultaneous recording of venous outflow. Without this it was difficult to ensure that there was no obstruction to the venous outflow by the oncometer pressing on the veins.

Radioactive measurements

Measurements of the uptake of radioactive ^{131}I during infusions of noradrenaline were made in two human subjects, and in one with adrenaline. In a further two subjects measurements of the blood level of radioactive protein-bound iodine (PBI) were made during infusion.

A dose of 20 μc of ^{131}I as iodide was given intravenously. Measurements of the activity of the thyroid were then made for a basal period of 90 min. After this time it was possible to extrapolate the curve for the next half hour with reasonable accuracy, during which time a standard infusion was given. In all cases this was sufficient to raise the systolic blood pressure to 180–190 mm Hg. Counting was continued for a further 30 min.

On the day following an injection of ^{131}I the subjects were given a further 30 min infusion and peripheral venous blood was taken before and after this for estimation of the radioactive PBI level.

In one dog, after an injection of 500 μc of ^{131}I given 24 hr previously, measurement was made of the radioactive PBI level in the blood issuing from the thyroid vein during basal periods and with infusions of noradrenaline.

RESULTS

Man

In forty-three out of forty-four subjects given noradrenaline noticeable increase in size of the thyroid occurred. This began within 5 min of starting

the infusion and persisted during its course. After stopping the noradrenaline infusion most of the swelling disappeared within 15–20 min, but some detectable swelling was present in a number of individuals for up to 24 hr. The mean increase in neck circumference was 1·3 cm and the largest 4·0 cm.

None of eleven subjects given adrenaline showed any increase in size of the thyroid.

In three of the subjects given noradrenaline a bruit was heard over the thyroid in the course of the infusion. For this reason it was thought that the increase in size might be due to an increase in blood flow. This is in accord with an observation on a patient with a phaeochromocytoma during an attack (Davis, Peart & van't Hoff, 1955).

TABLE 1. Effect of intravenous noradrenaline and adrenaline on thyroid blood flow in three human subjects

	Systolic B.P.			Change in thyroid blood flow (%)	
Subject	Basal	Ad.	NAd.	Ad.	NAd.
1	112	169	180	−11%	+26%
2	120	174	198	− 5%	+34%
3	104	206	171	−12%	+36%

Use was then made of the needle to measure the blood flow in the thyroid of one of us (W.S.P.) during a noradrenaline infusion. It was found that there was a considerable increase in blood flow, which returned to normal rapidly when the infusion was stopped. One subject then had measurements made with an infusion of adrenaline and a slight decrease in flow was noted.

In a further three subjects comparisons were then made of the effect of noradrenaline and adrenaline on the blood flow, with comparable blood pressure changes and closely similar dosage rates. Table 1 shows the means of the results obtained on these subjects. It will be seen that these confirm the initial findings. However, the increase in thyroid size outlasts the increase in flow by many hours, and thus the latter cannot be the only factor accounting for the increase in size.

Dogs

We were thus led to carry out the experiments in dogs where intra-arterial injections could be made and accurate measurement of volume made. Attempts to repeat on dogs the effects found in man were at first unsuccessful. It was found that either a decrease in flow or very little change occurred with intravenous infusions of both noradrenaline and adrenaline (Fig. 1*a*). However, these changes occurred, as will be seen, with only moderate elevations of blood pressure.

The possibility was therefore considered that the changes might be due to a greater increase in perfusion pressure, i.e. general blood pressure, assuming that noradrenaline was not a potent vasoconstrictor in the thyroid. The effects of intra-arterial infusions of noradrenaline and adrenaline were therefore studied (Fig. 1*b*).

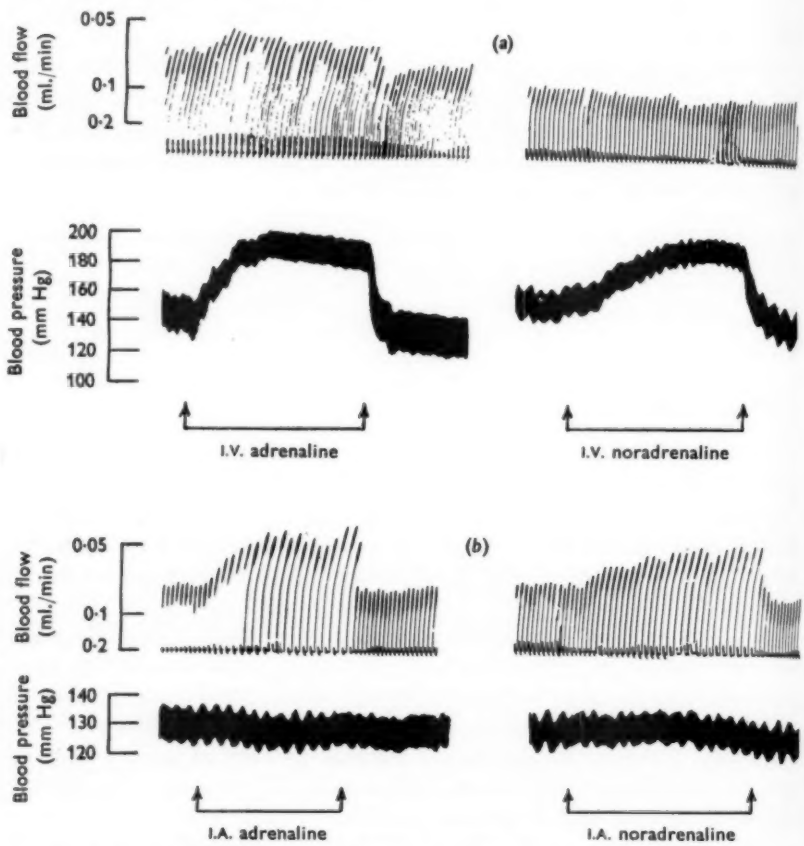

Fig. 1*a*. Effect of intravenous noradrenaline and adrenaline on thyroid blood flow in the dog. *b*. Effect of intra-arterial noradrenaline and adrenaline on thyroid blood flow in the dog.

It was found that adrenaline in all doses which were tried (0·01–1 μg/min) was constrictor, and produced a decrease in flow. In fact, with large doses the blood flow through the gland almost ceased.

Noradrenaline in large doses (0·1 μg/min) was also constrictor, but it was observed that at the start of the infusion, before the full effect was attained,

there was often a transient increase in flow. With smaller doses it was found that a rate of about 0·01 μg of noradrenaline per minute would produce a vasodilation in the thyroid in the three dogs studied in this way (Fig. 2).

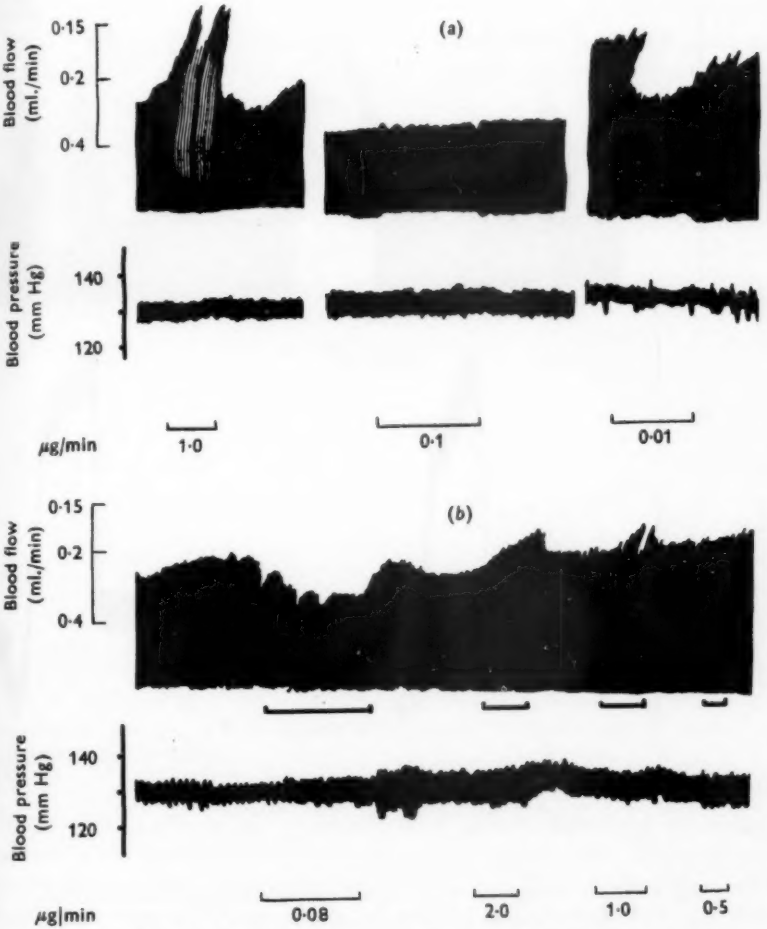

Fig. 2. Effect of intra-arterial noradrenaline on thyroid blood flow in the dog.

Since noradrenaline is known to be released at the majority of sympathetic nerve endings, it was considered possible that cervical sympathetic stimulation might produce an increase in flow. However, electrical stimulation of the common vagosympathetic trunk with an induction coil produced no significant change in thyroid blood flow in four dogs, although

contraction of the nictitating membrane was produced, showing the efficacy of the stimulation.

It has not been possible to show whether the increase in flow with intravenous noradrenaline is due to a small vasodilating dose reaching the gland,

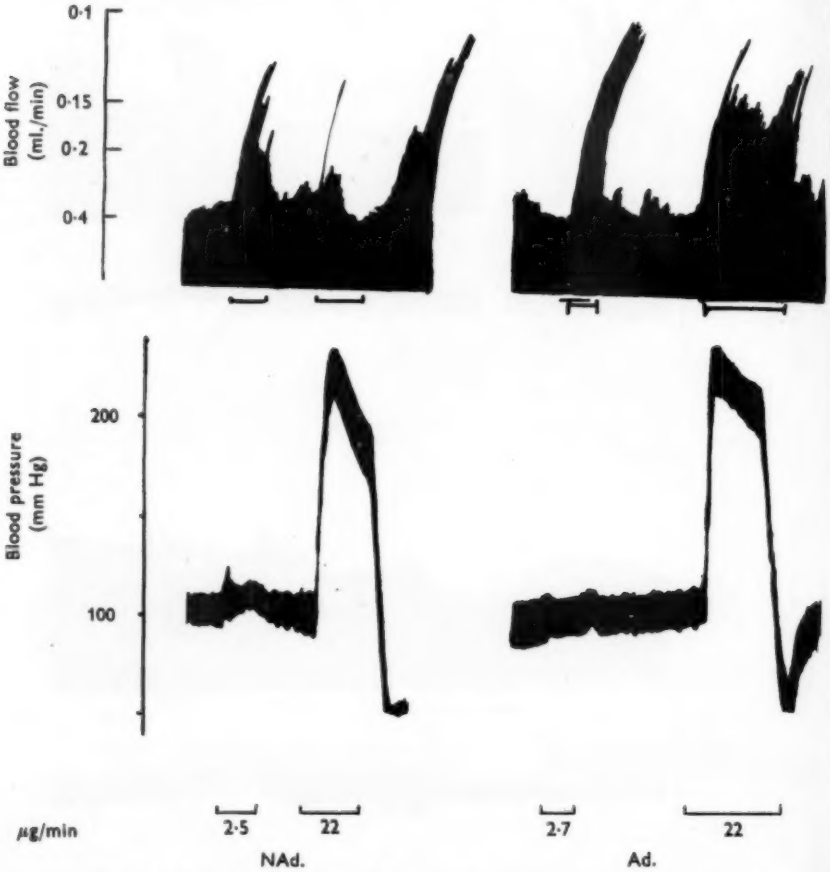

Fig. 3. Effect of large and small doses of intravenous noradrenaline (NAd.) and adrenaline (Ad.) on thyroid blood flow in the dog.

or merely to an increase in perfusion pressure. However, adrenaline in all doses (1–60 µg/min) given intravenously produced a decrease, but the large dose of noradrenaline (over 10 µg/min) increased the flow (Fig. 3). As the small dose did not do so it would seem most likely that an increase in perfusion pressure is the important factor. The same experiment carried out in man produced an identical result (Fig. 4).

Attempts to show a change in size of the dog thyroid during the infusion produced variable results. In one animal increases in size of up to 30% were shown with noradrenaline, but in three other animals decrease in size of the order of 2–3% occurred during infusion of noradrenaline and adrenaline.

Since it was considered possible that the increase in flow with noradrenaline might be associated with a change in function, attempts were made to measure functional changes during infusions.

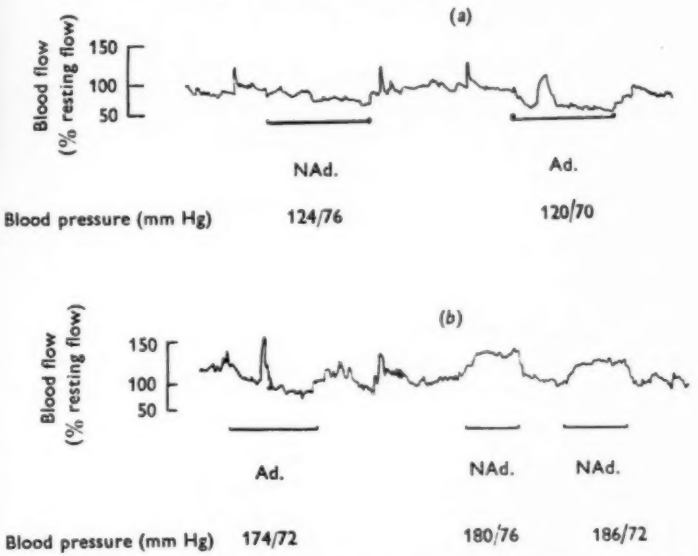

Fig. 4. Effect of large and small doses of intravenous noradrenaline (NAd.) and adrenaline (Ad.) on thyroid blood flow in man. (The upward spikes are artifacts due to swallowing.)

Radioactive studies

Studies were made in man of the effects of noradrenaline and adrenaline infusions on the uptake of ^{131}I and blood level of PBI, as already described. Figure 5 shows typical uptake curves obtained and Table 2 the PBI levels. The normal curve also shown in Fig. 5 flattens out after 90 min and it is possible to extrapolate the curve over the next 30 min. It will be seen that there is no detectable change in either parameter with infusions of noradrenaline. Again no change was found with adrenaline infusions. The blood levels of radioactive PBI were also not significantly altered during infusion of noradrenaline and adrenaline.

As changes in PBI would be shown more rapidly and in much greater degree in the thyroid than in systemic venous blood, an attempt was made

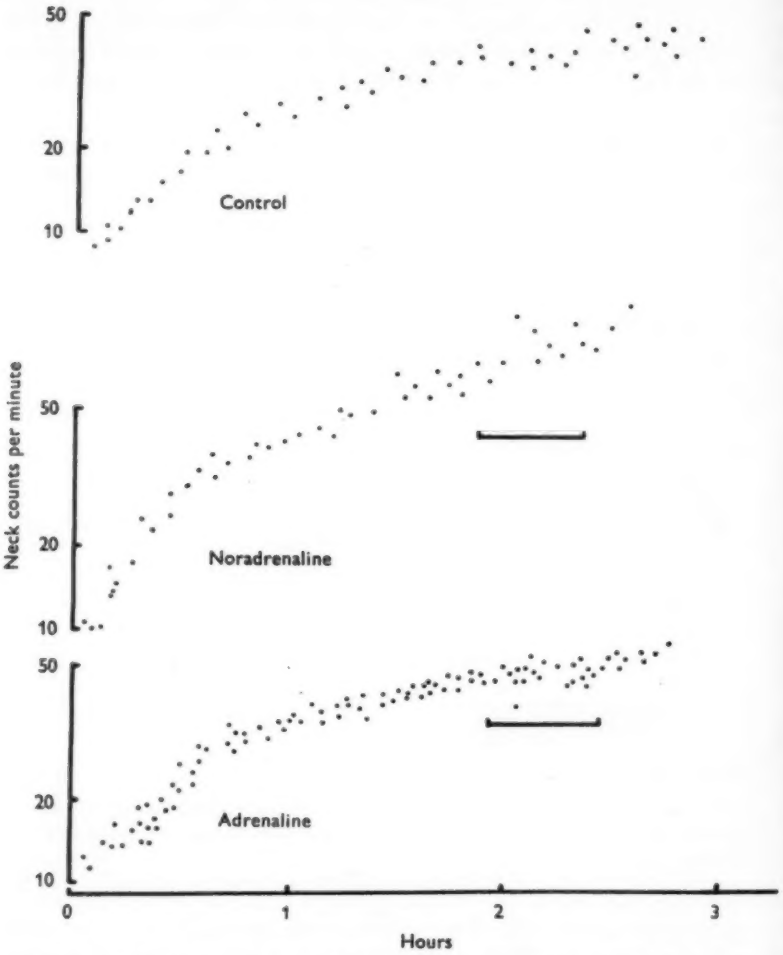

Fig. 5. Thyroid uptake curves following intravenous ^{131}I. The effects of intravenous noradrenaline and adrenaline are compared with a control subject.

TABLE 2. Radioactive protein-bound iodine levels in blood before and after noradrenaline infusion.

Subject	Protein-bound radioactivity (counts/min/15 ml. plasma; and S.D.) Before NAd.	After NAd.
S.T.	13·7 (2·01)	10·9 (1·98)
J.I.R.	16 (2·6)	10 (2·5)

to measure the output of radioactive PBI in the thyroid venous blood of the dog. It was found in the one dog studied that the concentration of activity in the blood fell as the flow rose during intravenous noradrenaline infusions, so that the total output per minute did not differ significantly from the control periods (Table 3).

TABLE 3. Radioactive protein-bound iodine levels in thyroid venous blood of a dog before and during intravenous noradrenaline

		Blood flow (ml./min)	PBI (counts/min/ml. blood; and S.D.)	PBI output (counts/min)
Period 1	Control	0·3	180·4 (7·3)	54
	NAd.	1·1	62·2 (2·8)	68·4
Period 2	Control	0·33	176·3 (7·0)	58·2
	NAd.	0·50	98·4 (3·6)	49·2

DISCUSSION

It has been possible to show an increase in size of the thyroid in only one anaesthetized dog with either intravenous or intra-arterial infusions of noradrenaline. In man swelling of the thyroid is the rule with noradrenaline, but not with adrenaline, and in both dog and man an increase in blood flow was observed. Increase in blood flow cannot be the sole explanation for the increase in size, however, since in the dog it was possible to increase the rate of flow without increasing the volume in three out of four animals. This may be allied with the observation of delayed disappearance of the swelling in man (up to 24 hr), which suggests oedema in the gland. A combination of arterial dilatation with some degree of venous constriction raising the capillary pressure and thyroid volume is possibly the correct explanation. Söderberg (1958) produced only a decrease in blood flow through the rabbit's thyroid with intra-arterial or intravenous noradrenaline, but did not show the increase we have noted. It is possible that he did not use small enough doses.

It is a surprising finding that there is a small dose of intra-arterial noradrenaline which is vasodilator, when all doses of adrenaline are vasoconstrictor. There seems to be only one other site in the body in which this is true. There is evidence that noradrenaline is a vasodilator of the human coronary arteries, where adrenaline has a constrictor effect (Smith, Syverton & Coxe, 1951). Much previous work has been confused by observations on the intact heart and not on the isolated coronary arteries (Wégria, 1951) and in these experiments the effect of the substances on the activity of the heart must be taken into account. One might consider that these dilator effects of noradrenaline might have a purpose, but if this is in some way to control the activity of the gland we were unable to show any effect on iodine uptake of the gland in two subjects. In the

one dog studied the output of radioactive PBI in the thyroid vein seemed to be inversely proportional to the blood flow, so that the output per minute was steady.

As the doses (20 μc) in man had of necessity to be small, it is not possible to say that either adrenaline or noradrenaline had no effect on the release of PBI into the circulation. The increase of activity in the peripheral blood which would be achieved by release from the gland is probably too small for satisfactory measurement. More detailed study of iodinated compounds coming out of the gland would be needed for certainty.

Since noradrenaline is released at most sympathetic nerve endings, it was expected that cervical sympathetic stimulation would affect thyroid blood flow; but there was no change in four dogs. A minimum of dissection was performed in two of these to avoid damage to the nerves. This suggests that the sympathetic innervation of the dog's thyroid is very slight and ineffective. Söderberg (1958) has shown that stimulation of the cervical sympathetic in the rabbit produced a decrease in blood flow and PBI output from the gland.

SUMMARY

1. Observations have been made on the increase in size of the thyroid with intravenous administration of noradrenaline. It is related closely, but not directly, to the increase in blood flow noted both in man and dogs.

2. In the dog small intra-arterial infusions of noradrenaline (about 0·01 μg/min) were vasodilator, but all larger doses and all doses of adrenaline were vasoconstrictor.

3. No change in ^{131}I uptake in two subjects or in protein-bound iodine blood levels in two further subjects was noted with intravenous infusions of noradrenaline or adrenaline. In one dog no change in the output per minute of radioactive protein-bound iodine in the thyroid vein was observed with similar infusions.

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THE DECARBOXYLATION OF AMINO ACIDS RELATED TO TYROSINE AND THEIR AWAKENING ACTION IN RESERPINE-TREATED MICE

BY H. BLASCHKO AND T. L. CHRUŚCIEL*
From the Department of Pharmacology, University of Oxford

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Recent work on the formation of catechol amines in mammals has shown that the amino acid L-DOPA (β-3:4-dihydroxy-L-phenylalanine) is the immediate precursor of adrenaline, noradrenaline and dopamine (β-3:4-dihydroxyphenylethylamine). DOPA is decarboxylated by DOPA decarboxylase, an enzyme present in many tissues, including chromaffin and nervous tissue (see Blaschko, 1959).

Although the molecule of DOPA is believed to be devoid of intrinsic pharmacological activity in intact animals, it has *in vivo* some sympathomimetic actions which are believed to be due to the catechol amines produced by the decarboxylation of L-DOPA. Also, in mice and monkeys tranquillized by reserpine, large doses of DOPA have a remarkable awakening effect (Carlsson, Lindqvist & Magnusson, 1957; Everett & Toman, 1959), caused probably by the catechol amines which are formed from it.

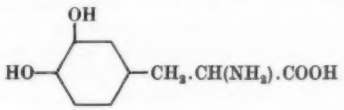
Much information is available on the substrate specificity of DOPA decarboxylase. This enzyme acts not only on 3:4-DOPA, presumably its natural substrate, but also on a number of other amino acids, mostly hydroxylated derivatives of phenylalanine. These observations, summarized elsewhere (Blaschko, 1950; Sourkes, 1955), have shown that the 2:5- and the 2:3-isomers of DOPA are readily decarboxylated by preparations of rat and guinea-pig tissues. L-Tyrosine is not decarboxylated, but its *meta*-hydroxy analogue (hereafter called metatyrosine) is a substrate of the mammalian enzyme, and it has recently been shown that in normal mice, rats, rabbits and dogs metatyrosine has excitatory actions, which are also thought to be due to the amine formed by the decarboxylation of the amino acid (Mitoma, Posner, Bogdanski & Udenfriend, 1957).

In the present study we have first tested the substrate specificity of the DOPA decarboxylase in the mouse and then examined a number of amino acids related to 3:4-DOPA for their awakening action in mice tranquillized

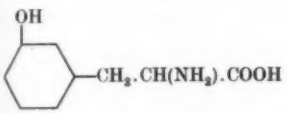
* Rockefeller Fellow.

by reserpine, including β-3:4-dihydroxyphenylserine, since this amino acid is slowly decarboxylated by the mammalian enzyme (Blaschko, Burn & Langemann, 1950).

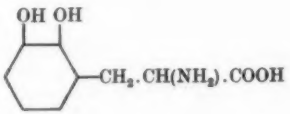
The formulae of the amino acids tested are shown below.



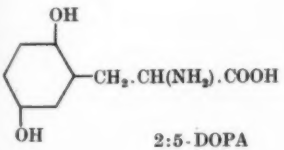
3:4-DOPA



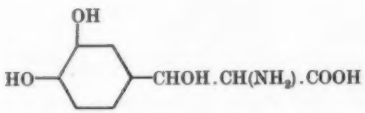
Metatyrosine



2:3-DOPA



2:5-DOPA



3:4-Dihydroxyphenylserine

METHODS

Substances used. The sample of *m*-hydroxy-DL-phenylalanine (metatyrosine) was that prepared by Dr H. R. Ing and used in an earlier study (Blaschko, Holton & Sloane Stanley, 1949); 2:5-dihydroxy-DL-phenylalanine was kindly given to us by Messrs Merck, Sharp and Dohme; 2:3-dihydroxy-DL-phenylalanine was a specimen prepared and previously used by Blaschko & Langemann (1951). The *threo*-3:4-dihydroxyphenyl-DL-serine, from Farbwerke Höchst, was a gift from Professor H. J. Schümann, Frankfurt a.M. The reserpine, given by Dr H. J. Bein, Basel, was dissolved in 20% ascorbic acid, to give a 0·5% stock solution which was further diluted for the injection.

Manometric determination of the DOPA decarboxylase activity. Mouse liver, kidney or brain were ground with sand in a cooled mortar and 0·067 M sodium phosphate buffer of pH 6·5 was added, 1 ml. for each gram of fresh tissue. The suspension was centrifuged at 27,000 *g* for 20 min and the slightly opaque supernatant fluid was used in the experiments.

The manometric procedure was similar to that described earlier (Blaschko, 1942), with 1·6 ml. of fluid in the main compartments of the conical manometer flasks and 0·4 ml. in the side bulbs. Initial substrate concentration after tipping was 2×10^{-3} M with L-amino acids as substrates, and 4×10^{-3} M with DL-amino acids. The temperature was 37·5° C, and the gas phase N_2. In some of the experiments 25 μg of the calcium salt of pyridoxal-5-phosphate was added to each flask in order to compare the rate of the enzymic reaction in the presence and absence of excess of co-decarboxylase. For the calculation of enzymic activity,

the initial linear part of the reaction was used; activities are expressed in terms of q_{CO_2}, i.e. μl. CO_2 formed by 1 mg of fresh tissue in 1 hr. In most experiments, the CO_2 retention was determined at the end of the experiment, but in the experiment with brain extract this was omitted.

Registration and evaluation of motor activity. We are indebted to Messrs D. Groves and O. B. Saxby, of this Department, for the construction of the cage used in the recording of motor activity of mice. The principle of the apparatus is shown in Fig. 1. A polystyrene box, $5 \times 5 \times 2\frac{3}{8}$ in. ($12{\cdot}7 \times 12{\cdot}7 \times 7$ cm), with a perforated lid, stands on a Perspex platform supported by four brass rods (diameter $\frac{1}{16}$ in. (1·6 mm), length $5\frac{1}{2}$ in. (14 cm)). A central rod is connected by a C-shaped spring wire, to a Rochelle salt crystal, and the output from the crystal is amplified in a two-stage amplifier and fed to a Kelvin–Hughes pen writing on a smoked drum. This arrangement responds to both horizontal and vertical movements of the animal.

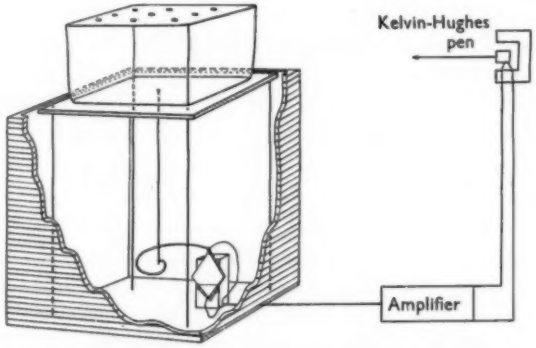


Fig. 1. Apparatus for recording motor activity of a mouse. For details see text.

For the evaluation of the results the tracing was varnished and a given length of it, for example, one equivalent to 10 min, was selected for measurement of its light transmittance with a selenium photo-electric cell. Results were expressed as percentage of normal activity, that is the mean of several readings taken from the same animal before the application of any drug.

RESULTS

Decarboxylation of phenylalanine derivatives by mouse tissue extracts

The DOPA decarboxylase activity was studied by using L-DOPA as substrate, both with and without added pyridoxal-5-phosphate. The results of these experiments on liver, kidney and brain are shown in Table 1.

It can be seen that the enzymic activity was highest in the liver and much less in the kidney and brain extracts. Using rat tissue extracts without added pyridoxal phosphate, Sloane Stanley (1949) found a mean q_{CO_2} for liver of 0·69 and for kidney of 0·07; in the mouse the corresponding figures are 0·51 for liver and 0·07 for kidney. In the mouse brain without added pyridoxal phosphate the q_{CO_2} was 0·06.

The addition of pyridoxal-5-phosphate (co-decarboxylase) *in vitro* did not significantly alter the rate of decarboxylation of L-DOPA by liver extracts but with kidney extracts the decarboxylation was significantly speeded up in most of the experiments, indicating that in the presence of added pyridoxal-5-phosphate the concentration of free co-decarboxylase was a rate-limiting factor (see Table 2). Using ox brain, Holtz & Westermann (1956) found an increase in the rate of decarboxylation when pyridoxal phosphate was added, but in our experiment with mouse brain there was no increase.

TABLE 1. DOPA decarboxylase activity of mouse tissue extracts. Substrate: 2×10^{-3} M L-DOPA (no pyridoxal phosphate added). Enzymic activity expressed as q_{CO_2} (μl. CO_2 formed by 1 g fresh tissue in 1 hr).

Tissue	No. of expts.	Mean value of q_{CO_2}
Liver	9	$0{\cdot}51 \pm 0{\cdot}16$
Kidney	7	$0{\cdot}07 \pm 0{\cdot}015$
Brain	3	$0{\cdot}06 \pm 0{\cdot}029$

TABLE 2. Effect of adding pyridoxal-5-phosphate on enzymic decarboxylation of L-DOPA in mouse tissue extracts

Tissue	Pyridoxal-5-phosphate omitted q_{CO_2}	Pyridoxal-5-phosphate added q_{CO_2}
Liver	0·52	0·54
	0·52	0·56
Kidney	0	0·13
	0·11	0·20
	0·12	0·13
	0·04	0·09
	0·03	0·04
Brain	0·09	0·03

No CO_2 formation occurred when L-DOPA was added to extracts of mouse spleen or heart (ventricles).

Substrate specificity of mouse liver decarboxylase. Since the liver was the most active source of enzyme in the mouse, we have used extracts of this tissue to study the decarboxylation of the other amino acids which was compared with that of L-DOPA.

Metatyrosine was readily decarboxylated by the mouse-liver enzyme, with a mean rate of 0·95, only slightly less than that of L-DOPA. The other two isomeric forms of dihydroxyphenylalanine were also readily decarboxylated, 2:3-DOPA at 0·87 times, and 2:5-DOPA at 0·91 times the rate of L-DOPA. These results show that the mouse-liver enzyme resembles that in rat liver and guinea-pig kidney (Blaschko *et al.* 1949; Blaschko & Langemann, 1951).

It may be mentioned here that earlier observations have not revealed any difference in the rates of decarboxylation of L-DOPA and DL-DOPA; it therefore seems likely that the rates of CO_2 formation from the racemic amino acids used in the present study represent the true rates of decarboxylation of the L-isomers.

Awakening effects of phenylalanine derivatives in mice

Normal animals

The intraperitoneal injection of 500 or 1000 mg/kg of L-DOPA in normal mice led to an increased activity of the animals for 1–4 hr, proportional to the dose administered. With 500 mg/kg the animals showed an increase in their normal motor activity and in their respiratory rate. With 1000 mg/kg of L-DOPA, over-activity was much more evident. About 30 min after the injection there was a great change in the behaviour of the animals: they jumped; they fought with each other; when touched, they attacked. Two of the animals went into convulsions. This over-activity lasted for about 4 hr. All the animals survived.

The effect of the amine oxidase inhibitor, iproniazid, was also studied by Carlsson *et al.* (1957). In agreement with their findings, an enhancement of the action of L-DOPA was observed after an injection of iproniazid. In one experiment, ten animals each received 500 mg/kg of L-DOPA alone. In another group of ten this was preceded by an injection of 100 mg/kg of iproniazid intraperitoneally. Five of these animals received the iproniazid 1 hr, the other five 2 hr, before the injection of L-DOPA. In all the iproniazid-treated animals the increase of motor activity was more pronounced and after about 2 hr the mice appeared to be exhausted. Of the ten mice treated with iproniazid, three died in the following 12 hr, whereas all the animals treated with L-DOPA alone survived.

Injections of D-DOPA (500 mg/kg) did not cause any increase in motor activity; if anything, the activity of the treated animals was less than that of the control animals.

Neither 2:3-DOPA nor 2:5-DOPA had an effect on motor activity in a dose of 500 mg/kg, but with 1000 mg/kg of 2:3-DOPA motor activity was diminished.

Metatyrosine (500–1000 mg/kg) caused an increase in motor activity of the mice similar to that caused by L-DOPA, although of somewhat less intensity. Like the response to L-DOPA, it started after about 30 min and lasted for 2–4 hr, as described by Mitoma *et al.* (1957).

Animals treated with reserpine

The effect of L-DOPA. The plan of the experiments was similar to that of Carlsson *et al.* (1957). To determine the appropriate dose of reserpine preliminary experiments were carried out with 10, 20, 30 and 40 mg/kg,

respectively, of reserpine, administered intraperitoneally. After 20 mg/kg of reserpine the mice were still spontaneously active, whereas after 40 mg/kg they had the appearance of heavily anaesthetized animals. A dose of 30 mg/kg was therefore chosen for the subsequent experiments and L-DOPA was given 16–18 hr after the reserpine; when iproniazid was also administered this was done 2 hr before the amino acid.

Groups of at least five mice were used for each dose level; the animals in each group were kept together in one cage. In addition, one animal was usually placed in the cage shown in Fig. 1, for records of its movements. The normal motor activity was recorded for at least 1 hr, just before administration of the reserpine. On the following day, the same animal was again placed in the cage, and records were taken both before and after the amino acid was administered intraperitoneally.

The awakening effect of L-DOPA observed in these experiments fully confirms the findings of Carlsson *et al.* (1957) and of Everett & Toman (1959). Before the amino acid was administered the mice all presented the appearance of being asleep, motionless and with eyelids closed. After the administration of L-DOPA the eyelids opened and within 20–30 min the animals resumed their normal motor activity. Then followed a period of over-excitement, during which the animals were more active than normal, untreated mice. After 3–5 hr the increased activity gradually disappeared and the animals returned to the motionless and sleeplike condition of the reserpinized control animals.

Of twenty-five animals treated with reserpine and subsequently with 500 mg/kg L-DOPA, one died. When iproniazid was also administered, four out of sixteen mice died. This increase in toxicity was probably related to the increased over-activity in the iproniazid-treated animals (see Figs. 2 and 3).

Amino acids related to L-DOPA. D-DOPA had no awakening action comparable to that of L-DOPA in reserpine-treated animals. However, 24 hr after 500 mg/kg of D-DOPA, seven out of eleven mice moved spontaneously and reacted briskly when touched, whereas in the control group treated with reserpine alone such a response was present in only two animals.

The two isomers of dihydroxyphenylalanine, 2:3-DOPA and 2:5-DOPA (1000 mg/kg), did not have any awakening action in the reserpinized animals even after iprioniazid.

When metatyrosine (500 mg/kg) was administered to the reserpine-treated animals, an awakening effect was seen similar to that of L-DOPA. A typical tracing is shown in Fig. 4, and the evaluation of another tracing from a similar experiment in Fig. 5. In the experiment of Fig. 5 almost 1 hr elapsed before spontaneous movements became evident. Under the influence of metatyrosine the animals resumed their normal activity, but

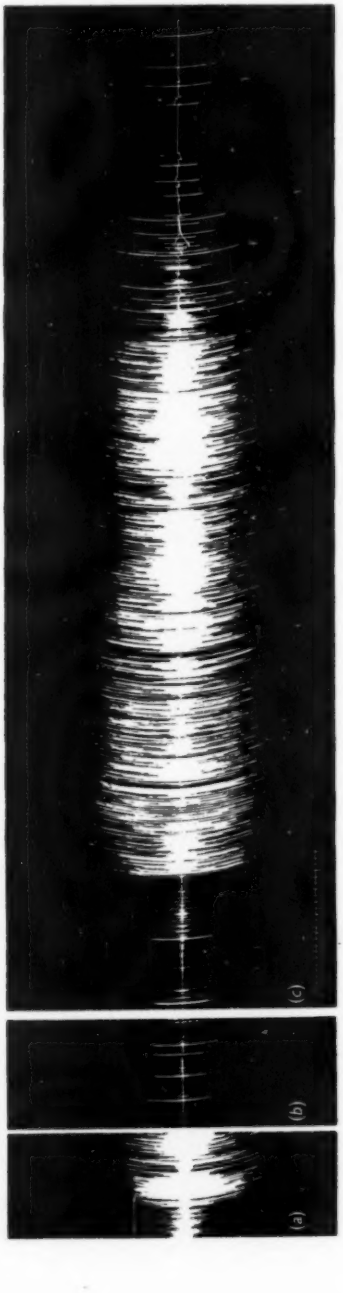


Fig. 2. Tracing taken with the apparatus of Fig. 1; effect of L-DOPA on the motor activity of a reserpine-treated mouse: (*a*) before reserpine; (*b*) 16 hr after 30 mg/kg of reserpine I.P. and 2 hr after 100 mg/kg of iproniazid I.P.; followed immediately by (*c*) after 500 mg/kg of L-DOPA I.P. Timemarker: 1 min.

this period was sometimes followed by one of over-activity. The effect of pre-treatment with iproniazid was less pronounced than in the experiment with iproniazid and L-DOPA, where the characteristic signs of over-activity were always stronger after pre-treatment with iproniazid. However, when a larger amount of metatyrosine was given (1000 mg/kg), the enhancement of the effect of the amino acid by iproniazid was more evident.

Although the action of metatyrosine on the reserpine-treated mice was similar to that of L-DOPA, the onset of the awakening action was somewhat slower and the effects were less pronounced, particularly in the absence of iproniazid. The action of metatyrosine began to decline after 2–3 hr until the animals no longer differed from the reserpine-treated controls.

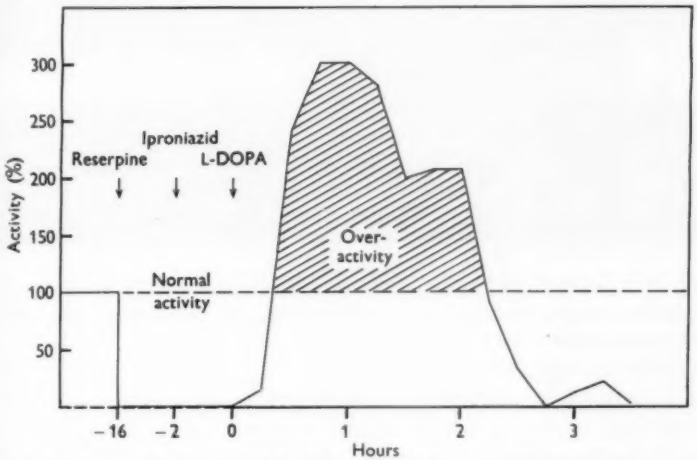


Fig. 3. Evaluation of motor activity of a reserpine-treated mouse after administration of iproniazid (100 mg/kg) and L-DOPA (500 mg/kg), as in the experiment shown in Fig. 2, the level of activity before reserpine (30 mg/kg) being taken as 100%. This animal showed no changes in spontaneous activity in the period of observation before reserpine administration.

Enough *threo*-3:4-dihydroxyphenylserine was available for an experiment in which 500 mg/kg of the amino acid was administered to three reserpine-treated mice. Almost immediately the ptosis of the eyelids disappeared, the respiration rate increased, and in two of the three animals tremors were seen that persisted for 2 to 3 hr.

The rapid onset of these symptoms makes it necessary to consider the possibility that the effects seen were due, at least in part, to a small contamination of the sample with noradrenaline; it is known that samples of adrenaline carboxylic acid, the corresponding *N*-methyl amino acid, are contaminated with adrenaline (see Schmiterlöw, 1951).

No awakening effect of dihydroxyphenylserine was seen.

Dopamine and noradrenaline. Dopamine, the product of the decarboxylation of DOPA, has been tested in mice treated with reserpine and iproniazid, in doses of 500 mg/kg of the hydrochloride. About 3 min after injections of the amine all the mice opened their eyes; the respiration rate was raised for 2–3 hr, and there was a flow of tears, but apart from occasional tremors there was no return of normal motor activity.

Reserpine-treated mice, when injected with 5 mg/kg of noradrenaline bitartrate intraperitoneally, opened their eyes and righted themselves

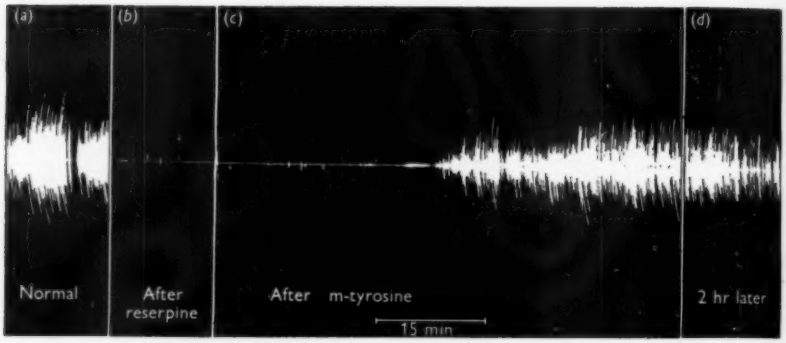


Fig. 4. Effect of metatyrosine on a reserpine-treated mouse. Motor activity (*a*) before reserpine; (*b*) 16 hr after reserpine (30 mg/kg); (*c*) immediately following, after metatyrosine (500 mg/kg); (*d*) 2 hr later.

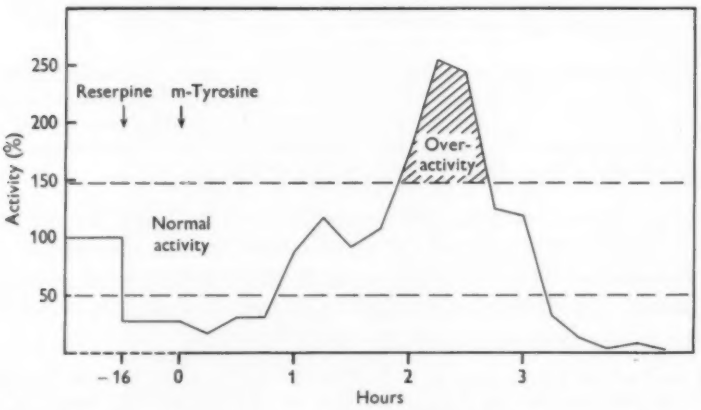


Fig. 5. Effect of metatyrosine on the motor activity of a reserpine-treated mouse. Doses as in the experiment of Fig. 4. This animal showed some changes in spontaneous activity before reserpine.

almost immediately, but did not return to their normal motor activity. With 50 mg/kg there were also occasional tremors, but no return of normal motor activity.

DISCUSSION

The results suggest that the amino acids which were able to awaken the mice treated with reserpine acted after being converted to the corresponding amines. This is supported by the following observations. There was always a time lag between administration of the amino acids and onset of action. Also, D-DOPA, which is not a substrate of the decarboxylase, was inactive as an awakening agent, except for an occasional feeble response 24 hr after its administration; this is of interest, since Holtz & Credner (1944) have found that in the rat D-DOPA can be converted to L-DOPA, probably through a transamination reaction involving 3:4-dihydroxyphenylpyruvic acid.

The different responses of the animals to an injection of the amines and that of the amino acids have been interpreted as due to an inability of the amines to penetrate what is called the blood–brain barrier. It is believed that the amino acids are able to pass this barrier and are then converted to the pharmacologically active amines in the brain, in close vicinity to their sites of action. This view is supported by findings that the administration of DOPA leads to an accumulation of catechol amines in the brain (Raab & Gigee, 1951) and that of metatyrosine to the appearance of metatyramine (Mitoma *et al.* 1957).

Where is this barrier situated? It is impossible to give a precise answer to this question, but it is probably not an anatomically defined barrier that limits the concentration of amine after systemic application. Central actions of adrenaline and other sympathomimetic amines, e.g. upon the electric activity of the brain, are well known. Also, there have been reports that tritium-labelled adrenaline can be recovered from the hypothalamus (Weil-Malherbe, Axelrod & Tomchick, 1959) which must therefore be accessible to adrenaline.

The amino acids differ from the corresponding amines in that the latter only are substrates of amine oxidase. Amine oxidase, together possibly with O-methyl transferase and other enzymes active in amine metabolism, would prevent the amines but not the amino acids from reaching the excitable structures. The acids are decarboxylated near their sites of action, and in this way an effective concentration of amine is built up.

The enhancement by iproniazid of the awakening action of L-DOPA, as well as the higher mortality, is of interest in view of recent work on the inactivation of catechol amines. It seems doubtful whether amine oxidase determines the time course of action of noradrenaline or adrenaline, but there can be little doubt that it is an important factor in the inactivation

of dopamine (Blaschko, 1956). An increased accumulation of dopamine might either have intrinsic awakening action or speed up the formation of noradrenaline and adrenaline.

That L-DOPA is the normal precursor of the catechol amines in the body is now generally accepted (see Blaschko, 1959). Its effects upon the reserpinized animal are so profound that it is tempting to assume that the catechol amines are in some way essential for the maintenance of wakefulness in the normal animal. Other observations support this view, e.g. the awakening actions of amphetamine and of other sympathomimetic compounds. It is of interest that these effects are most pronounced in amines immune from attack by amine oxidase. Further, there are well-known 'arousal' reactions caused by adrenaline and related compounds, and the changes in the electroencephalogram resemble those seen in wakefulness (see Rothballer, 1959).

L-DOPA is a substance normally occurring in mammals, but other substances may also have awakening actions in reserpine-treated animals. Gaddum & Vogt (1956) have found that in cats morphine administered intraventricularly counteracts the effects of reserpine given intraperitoneally. However, in mice, morphine did not produce effects comparable to those produced by L-DOPA (Chruściel, unpublished observation).

The decarboxylation of 3:4-dihydroxyphenylserine by the mouse-liver enzyme was not tested, but it is known that the acid is slowly decarboxylated by the mammalian decarboxylase (Blaschko *et al.* 1950; Holtz, 1959). On decarboxylation the acid would give rise to noradrenaline, bypassing the dopamine stage. If the serine derivative had produced awakening effects comparable to those of L-DOPA, this might have been valuable as an indication that noradrenaline rather than dopamine was responsible for the effects observed after giving L-DOPA. The relatively low efficacy of the serine derivative need not necessarily be due to the omission of the dopamine stage; it might well be due to the slow rate of decarboxylation of this amino acid.

Although metatyrosine is not normally found in the organism, it is able to awaken reserpinized mice. Mitoma *et al.* (1957) have already noted excitatory actions of this compound in normal mice. The amino acid is readily decarboxylated by the mammalian enzyme *in vitro* and presumably *in vivo*.

How do the excitatory actions of metatyrosine arise? It seems likely that the first step is again the formation of the corresponding amine. It is conceivable that metatyramine is converted to dopamine by the introduction of a second phenolic hydroxyl group, but there is nothing to suggest that a reaction of this kind can occur in the mammalian body. Other alternatives are either an intrinsic pharmacological action of metatyramine

exerted on the structures normally excited by the catechol amines, or an action mediated through an increase of the catechol amines normally formed in the body, either by their release or by an interference with their inactivation. In the reserpinized animal, where the stores of catechol amines are depleted, a release seems unlikely but can at present not be definitely excluded. The possibility that metatyramine itself has intrinsic pharmacological activity is worth considering, since it is known that phenylethylamine derivatives hydroxylated in the *meta* position are usually more potent sympathomimetic agents than the *para*-hydroxy compounds (see Bovet & Bovet-Nitti, 1948). An intrinsic awakening action of metatyramine would be of interest since it is a compound not normally occurring in the body.

That rapid decarboxylation alone is not a sufficient requisite for an awakening action is shown by the absence of any awakening effect with 2:3-DOPA or 2:5-DOPA which are both rapidly decarboxylated. This suggests that an amino acid must satisfy two specificity requirements for the awakening effect, first, that of the amine-forming enzyme, and secondly, that of the excitable structures upon which the amines act.

SUMMARY

1. The activity of the DOPA decarboxylase of the mouse on a number of amino acids related to tyrosine has been measured.

2. Extracts of liver, kidney and brain decarboxylated not only L-DOPA, but also metatyrosine, 2:3-DOPA and 2:5-DOPA.

3. The effect of the intraperitoneal administration of a number of amino acids on the motor activity of normal and reserpine-treated mice was studied.

4. A method for the evaluation of the motor activity is described.

5. L-DOPA and metatyrosine had strong awakening effects upon mice treated with reserpine.

6. D-DOPA, 2:3-DOPA, 2:5-DOPA and *threo*-3:4-dihydroxyphenylserine had no awakening actions in reserpine-treated mice.

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DYNAMICS OF ACCOMMODATION RESPONSES OF THE HUMAN EYE

BY F. W. CAMPBELL AND G. WESTHEIMER*
From the Physiological Laboratory, University of Cambridge

(*Received* 14 *October* 1959)

It is not till recently that an optometer has become available (Campbell, 1956; Campbell & Robson, 1959) that enables one to obtain continuous, high-resolution records of changes in the refractive power of the human eye. Using this optometer we have investigated the nature of the accommodation responses when young emmetropic subjects were presented monocularly with a variety of focus-stimulating conditions.

METHODS

Recording

The recording optometer used in these experiments is a modified version of the instrument described by Campbell & Robson (1959). A diagrammatic representation of the optometer is shown in Fig. 1; it is not to scale.

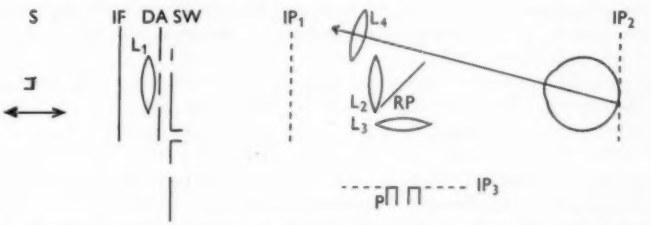

Fig. 1. Schematic diagram to illustrate optical system of optometer (not to scale). S, filament light source. IF, infra-red filter. L_1, L_2, L_3, L_4, lenses. DA, double aperture. SW, sector wheel. IP_1, IP_2, IP_3, image planes. RP, reflexion plate. p, photocells.

The light source (S) consists of a V-shaped filament automobile headlamp. Lens L_1 has a double slit diaphragm (DA) placed immediately before it. Close to this diaphragm is placed a slotted sector wheel (SW) which interrupts the two beams, formed by the slits, alternately at a frequency of 300 c/s. Lens L_1 forms an image of the V-filament in the plane IP_1. Lens L_2 collects the two beams diverging from this plane and forms an image of the double aperture DA in the plane of the pupil of the subject's eye. If the subject were to look directly into the optometer he would observe a sharp image of the filament S provided his retina (IP_2) was conjugate to the image plane IP_1. If his retina is not conjugate to this

* Present address: The Ohio State University, Columbus 10, Ohio, U.S.A.

plane he would observe a double image of the filament. The light source S is so arranged that it can be moved along the axis of the optical system. In this way it is possible to place the image plane IP_1 in such a position that a single image can be formed on the retina, even allowing for abnormal ocular refraction.

By placing a plate RP with a reflexion:transmission ratio of 0·5 before the eye at 45° to the axis of the optometer, it is possible to collect the small fraction of the light reflected from the fundus which passes out of the eye through the pupil. A real image of the reflected fundal image of the filament can then be formed by lens L_2 in plane IP_2. This real image is allowed to fall on a pair of germanium photodiodes (Siemens Type TP 50) placed side by side. The power of lens L_2 is so chosen that the size of the images of the V-filament is similar to that of the photosensitive surfaces of the photodiodes. The diodes are so aligned that a limb of the V-shaped filament image falls on each photosensitive surface. As the photodiodes are sensitive to infra-red radiation, an infra-red filter (Ilford No. 207) is placed in position IF to render the light invisible. The electronic arrangement is identical with the one already described (Campbell & Robson, 1959). The electronic filter used in most of the present experiments has a corner frequency of 3·3 c/s with a 12 db/octave slope. Critical findings were checked on records obtained with a filter with corner frequency of 33 c/s and a 18 db/octave slope. Except for the presence of high frequency noise the latter records do not differ materially from those shown in this paper. The phase lag of both filters in the frequency range important in the present study is small.

The modifications of the original design of the optometer have resulted in the following advantages:

1. The photodiodes now used do not require cooling with solid CO_2 as did the lead sulphide cells in the original instrument.

2. The internal photoamplification and output from the diodes is higher than that from the lead sulphide cells and consequently the design of the first stage of the amplifier is not so critical.

3. The photosensitivity of the diodes is better matched to the spectral distribution of the source, the transmission of the infra-red filter and the absorption characteristics of the eye media. As a result, the instrument is less sensitive to ambient room light and, indeed, can be used in an undarkened room.

4. The original optometer used a reflexion cube in place of the reflexion plate in the present instrument. The cube was found to allow stray light, arising from interface reflexions of the ingoing beams, to enter the photocell optic system and thus reduce the signal-to-noise ratio of the instrument. The reflexion plate at present in use is of the dielectric type (Barr and Strouds Ltd., Glasgow) with a total absorption loss of less than 2% compared with over 30% in reflexion plates coated with silver or other metals.

5. By using the image of the filament itself in the retinal plane instead of the image of a slit diaphragm, it has been possible to eliminate one lens in the optical system with a resulting further increase in light flux entering the eye.

The optometer was calibrated by requesting experienced subjects with good voluntary control over their accommodation to focus on a series of targets, placed at known distances from the eye, until no trace of out-of-focus blurring could be detected. From these measurements, it was concluded that the optometer and its recording system was linear to within ±10% over a range of 3 dioptres.

Stimulating

In addition to the above accommodation recording system, a visual stimulating system (Fig. 2) was placed before the same (left) eye of the subject. It consists of two illuminating beams brought together by a beam splitter K. Each beam has a neon lamp (Hivac Type CC8L) N as a source which illuminated from behind an opal glass screen T limited by an aperture A. On each opal glass screen a black disk was printed which subtended 10′ at the

eye. Both beams are adjusted carefully to appear exactly in line when viewed by the subject through lens L_4 placed on the subject's side of the beam splitter.

The power F (in dioptres) and the position of this lens are so chosen that its second focal plane is in the nodal point of the subject's eye. Under this condition, if x is the displacement of the target from the first focal plane of the lens in metres, we have, by Newton's lens formula,

$$xx' = f^2, \tag{1}$$

where $f(= 1/F)$ is the focal length of the lens in metres and x' is the distance in metres between the image of the target and the nodal point of the subject's eye. If we now express this distance x' in terms of its reciprocal D, equation (1) becomes

$$D = xF^2.$$

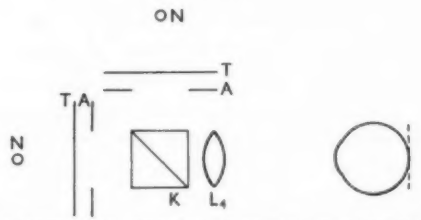

Fig. 2. Schematic diagram to illustrate the optical system used to present targets to the eye (not to scale). Neon lamps N transilluminate opal glass screens T each containing a small high-contrast target and limited by apertures A. The two beams are brought together by beam splitter K and are viewed by the eye through lens L_4. One source N, target T, and aperture A can be moved as a unit to vary the accommodation stimulus.

Here F is the dioptric power of the lens L_4—a constant under our experimental conditions—and x is the distance in metres between the target and the first focal plane of the lens. D is the dioptric distance between the image of the target and the nodal point of the subject's eye and is thus the measure of the accommodation required by the subject to image the target T, as seen through lens L_4, sharply on his retina. The advantage of this experimental arrangement is that D is now a linear function of x, the target displacement. Another feature of this optical system is that the retinal image size and the retinal illuminance of the target are independent of D.

The two neon lamps are connected to a high-speed relay and the experimenter can, by means of a switch, change from one beam to the other practically instantaneously. A photocell placed close to one of the neon lamps serves to indicate on one of the channels of the pen recorder the instant of the change-over. The luminance of each of the two illuminated fields as seen by the subject through the beam splitter and the lens L_4 is 80 cd/m^2. The neon light source, opal glass screen and aperture of one of the beams can be moved as a unit either by a rack and pinion movement or by an attachment which pulls them against elastic bands by a string fixed eccentrically on a wheel rotated by a variable speed motor.

In some experiments lens L_4 is removed. The subject then views the opal glass screens directly through the beam splitter. In this case when there is a change-over from one beam to the other and there is a difference in distance of the two targets from the eye, there is a change not only in focus but also in apparent size. The dioptric measure of the required change in accommodation is the difference between the reciprocals of the target distances in metres.

The stimulating system is placed at an angle of 20° with the recording system. Thus when the subject has his eyes positioned to view the targets, the optometer light—infra-red and, therefore, invisible—is imaged in the periphery of the retina.

Procedure

Accommodation responses were recorded in six emmetropic subjects between 20 and 40 years of age. The left pupil was dilated by instilling, about 20 min before the recording session, one or two drops of 1% paredrine into the conjunctival sac. The subject was placed before the apparatus using a bite bar and forehead rest to ensure steadiness of the head. The right eye was occluded. It was ascertained that a change-over from one stimulating beam to the other did not produce any eye movements. The subject was then instructed to keep the target clear at all times. A recording session lasted 10–15 min.

RESULTS

Refractive changes were recorded in response to the following stimulus changes: (*a*) instantaneous displacement of a target from one optical viewing distance to another with and without size cues (step stimulus), (*b*) instantaneous displacement of a target from one optical viewing distance to another with a quick return to the original distance (rectangular pulse stimulus), (*c*) simple harmonic oscillations of a target through a focus range and (*d*) gradual change in focus of an object, all other characteristics of the object remaining unchanged. We also recorded voluntary accommodation changes, i.e. accommodation movements initiated voluntarily by the subject without the occurrence of any changes in the visual field.

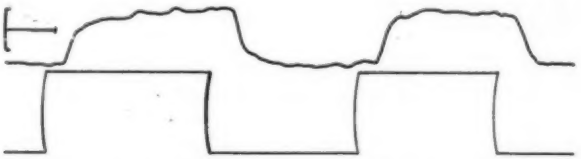

Fig. 3. Record of accommodation responses to a 2 D step stimulus and return to zero level of accommodation (subject F.W.C.). Allowance should be made for the arc of the pen. Top line, accommodation (length of horizontal line, 1 sec; height of arc, 1 D): upward movement represents far-to-near accommodation. Bottom line, stimulus signal, same scale. This record is an example of single-sweep accommodation responses.

Steps stimulus. Two typical responses to this kind of stimulus when size cues were present are illustrated in Fig. 3. There is a reaction time and the response occurs in a single sweep. The same kind of record is also obtained when the subject is asked to accommodate voluntarily. The maximum velocity reached during a 2 D movement is of the order of 10 D/sec. There is an increase in the maximum velocity with increase in the extent of the movement, but we have not yet studied this relationship systematically since we find it difficult to record single-sweep accommodation responses exceeding 3 D, other than voluntary ones.

When the only cue to accommodation is blur, it is more usual to see movements like those illustrated in Fig. 4. There is a considerable vari-

ability in the form of the responses and one can often distinguish more than one step.

All accommodation responses follow the corresponding stimuli after a reaction time. The mean and standard deviations of ten measurements of reaction time for far-to-near accommodation and for near-to-far accommodation in each of our six subjects are shown in Table 1.

Fig. 4. Typical accommodation responses to a 2D step stimulus and return when targets change only in focus and not in size (subject F.W.C.). Note variability in response form. Length of horizontal line, 1 sec; height of arc, 1D.

TABLE 1. Reaction time (sec) of accommodation responses to sudden changes in focus

	Far-to-near accommodation		Near-to-far accommodation	
Subject	Mean	Standard deviation	Mean	Standard deviation
F.W.C.	0·30	0·09	0·38	0·07
H.C.	0·36	0·16	0·27	0·08
J.G.R.	0·30	0·05	0·33	0·08
R.R.	0·42	0·14	0·46	0·13
P.G.	0·42	0·07	0·42	0·07
D.H.F.	0·36	0·06	0·45	0·06
Average	0·36	0·09	0·38	0·08

When the response is carried out in a single sweep, the movement time appears to be independent of the amplitude of the movement. Average values obtained for movement time are 0·64 sec for far-to-near accommodation and 0·56 sec for near-to-far accommodation. The total time which elapsed between the onset of a stimulus to change accommodation and the achievement of a reasonably stable level of accommodation near the new requirement depends upon the mode of response. When there is a single-sweep response it is about 1 sec, but when the response has more than one phase the elapsed time may be as much as 2 sec or more.

Rectangular pulse stimuli. Figure 5 illustrates a series of responses when a far visual stimulus is replaced for various brief periods by a stimulus at a nearer optical distance. It is seen that corresponding to both the ascending and the descending limbs of the stimulus pulse there is an ascending and a descending accommodation change. The interval between the onsets of these accommodation changes is very much the same as the

duration of the stimulus pulse, certainly within the limits of the variability of reaction time shown in Table 1. Since the time course of an accommodation movement is relatively slow, the form of the response pulse depends on the pulse duration, the return action commencing during various stages of completion of the original movement. Pulses momentarily exposing a target more remote than the original and final targets yield responses which on the whole show similar characteristics although there are then occasional pulses without concomitant responses.

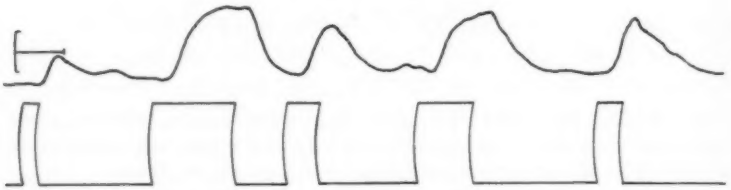

Fig. 5. Accommodation responses when a far visual stimulus is replaced by an identical one at a nearer optical distance for various time intervals presented in random order (rectangular pulse stimuli). Subject J.G.R. Length of horizontal line, 1 sec; height of arc, 1D.

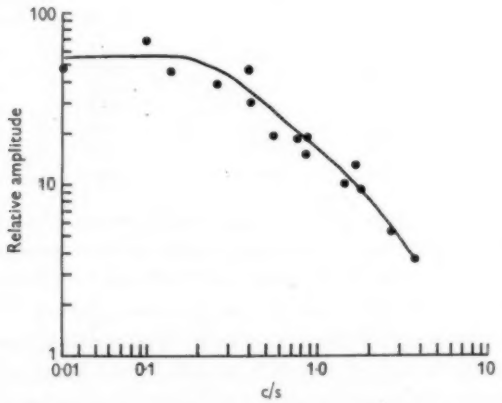

Fig. 6. Graph illustrating change in amplitude of accommodation response when a target is oscillated sinusoidally through a constant focus range of 0·6 D at various frequencies (subject D.D.). Ordinates, relative amplitude of response oscillations; abscissae, frequency of stimulus oscillations. Electronic filter attenuation has been allowed for. Log scales.

When the pulse duration becomes as short as 100 msec or less, responses are small or sometimes even absent. Pulses of duration less than 80 msec were not studied.

Fig. 7. Accommodation response as a target's optical distance is gradually changed (subject F.W.C.). Length of horizontal line, 1 sec; height of arc, 2D.

Fig. 8. Accommodation fluctuations when a subject (H.C.) is unable to return to zero level of accommodation. Height of arc, 1D.

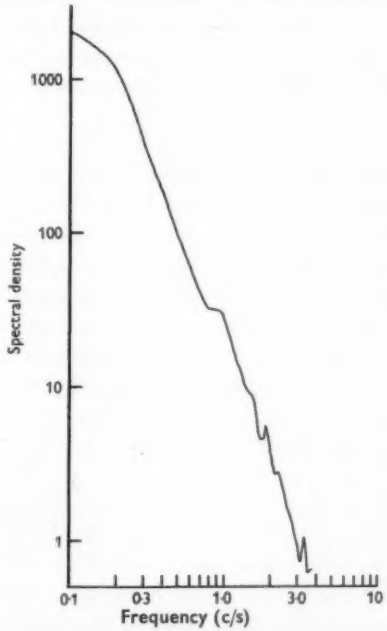

Fig. 9. Spectral density of fluctuations of which Fig. 8 is a short typical section. Ordinates, relative spectral density; abscissae, frequency (c/s). Log. scales.

Simple harmonic stimuli. When a visual target is oscillated to produce focus settings (measured in dioptres) varying sinusoidally with respect to time, the accommodation shows oscillations of the same frequency. The amplitude of the response oscillations decreases as the frequency increases (Fig. 6). There is always a time lag between peaks in the stimulus and corresponding peaks in the response and this is found to lie between 360

and 500 msec. The maximum frequency of stimulus oscillation at which there is any evidence of a concomitant response is about 4 c/s. For slow oscillations the response is not smooth; step discontinuities and superimposed fluctuations are evident. Records of accommodation changes in response to sinusoidal focus changes are contained in a previous communication (Campbell, Robson & Westheimer, 1959, Fig. 8).

Gradual change in optical viewing distance. A response to such a situation is illustrated in Fig. 7. It usually does not follow the stimulus gradient smoothly but contains discontinuities and fluctuations. The record also demonstrates that a subject may fail to match the target velocity correctly and may make movements in the incorrect direction. Occasionally a subject's accommodation will not relax to infinity when a target is moved from near to optical infinity in our instrument. The accommodation may then fluctuate around a mean level of about 1 D for many seconds. Figure 8 illustrates a section of an accommodation record in such a case and Fig. 9 the spectral density of the fluctuations.

DISCUSSION

The reaction time for an accommodation response is considerably longer than that for the pupil, 0·26 sec (Kawahata, 1954), or for eye movements, minimum of 0·12 sec (Westheimer, 1954*a*). The high sensitivity of the optometer enables us to determine the onset of a response to better than 0·1 D so that it is unlikely that the values of reaction time given here are over-estimated through the inclusion of some movement time.

Is the difference in reaction time between accommodation and the other oculomotor systems due to a difference in peripheral response mechanism or should it be interpreted as indicating a longer central delay? The pupil response to light is predominantly if not entirely due to contraction of the sphincter muscle of the iris and has a close affinity to the far-to-near accommodation response in the neural and muscular aspects of the effector system. Both responses are mediated by the third nerve parasympathetic pathway via the ciliary ganglion, and a smooth muscle system activated by acetylcholine. The pupil, too, shows a response when the subject looks at a near object—the so-called near reflex of the pupil. There is little doubt that, while the pupillary light reflex follows an uncomplicated pathway involving the pretectal area, the near response of the pupil shares with the accommodation and convergence reaction a central pathway capable of making distinctions between and interpretations of retinal images which we usually associate with cortical areas (Campbell & Westheimer, 1959).

On this basis one would expect the pupillary response to a near target to have a latency longer than that of the simple pupil reflex to light, in fact, a latency comparable with that of the accommodation response to

a near target. We have carried out measurement of the latency of the pupil response to a near target in one subject (F.W.C.) by presenting targets at two optical distances in our stimulus beams and changing over instantaneously from one to the other, ensuring that no brightness differences but only a focus difference existed between the two beams. There was a mean latency of 0·32 sec for the pupil response to a near target, compared with a mean latency of 0·30 sec for an accommodation response to a similar stimulus. Pupil responses to light measured in the same subject at the same session had a mean latency of 0·24 sec. We conclude that the difference in latency of the two kinds of pupil response is an indication that in the pupil response to a near target there is a longer central component, the retinal response time and the peripheral effector time being comparable in both classes of pupil response.

The latency of a convergence response is about 0·20 sec (Westheimer & Mitchell, 1956). Making the assumption that the convergence, accommodation and pupil responses to a near target share the same afferent and most of the central pathways, we conclude that the longer latency of the last two responses is a consequence of a long-latency component in the peripheral accommodation and pupil effector mechanisms.

Comparison of Figs. 3 and 4 makes it clear that the latter includes responses that are not maximal. Several step-like responses may sometimes be made, generally separated by time intervals of the order of a reaction time. A significant observation is that when the targets differed only in focus and not in size, the responses differed from stimulus to stimulus even within a few seconds and no systematic modifications could be detected during the several dozen responses recorded in a session. We have to conclude, therefore, that while the accommodation mechanism is capable of giving single-sweep, relatively rapid responses, even an instantaneous change-over of optical distance of the target does not assure that such a response will supervene. In a study of the mechanics of the peripheral response mechanism attention must clearly be given to this difficulty.

This variability of response is also seen in accommodation from near to far where discrete phases and oscillations may be distinguished. Using a technique of recording the Purkinje–Sanson image to estimate accommodation, Kirchhof (1940) also reported variability in the response form, although he did not demonstrate the details of these variations. His average values for movement time, viz. 0·5 sec for far-to-near accommodation and 0·43 sec for near-to-far accommodation, are of the same order of magnitude as we have found.

The responses to pulse stimuli afford insight into another aspect of the accommodation mechanism. Pulses as short as 100 msec may provoke a

response even though the stimulus then had returned to its original state more than 200 msec before the onset of the response. The upward response to a pulse stimulus will proceed only for a time interval of the order of the pulse length before a return of accommodation commences. We have evidence here that we are dealing with a continuous monitoring system and that an accommodation movement can be halted during its progress. This may be contrasted with the behaviour of the extraocular muscle system where pulse stimuli, if they yield a response, produce saccadic movements which cannot be modified during their progress (Westheimer, 1954*b*).

The fact that information about the state of focus is assimilated during an active accommodation movement is particularly well illustrated in Fig. 10. Here the return of the pulse stimulus occurs during the early stages of the response to the upstroke of the stimulus. Nevertheless, the response return occurs without further delay, a reaction time after the stimulus return.

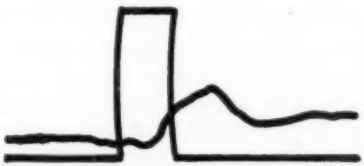

Fig. 10. Accommodation response to a 2D pulse stimulus of 0·32 sec duration (subject H.C.).

The form of the single-sweep accommodation responses is to a first approximation exponential with an average time constant of about 0·25 sec and may, therefore, be regarded as the response of a first-order system (a single stage, resistance-capacitor, circuit model) to a step input. Such a model subjected to an amplitude *versus* frequency analysis would yield a curve similar to that shown in Fig. 6. Working with this simple model one would expect a frequency-dependent component in the phase relationship between stimulus and response in addition to a non-frequency dependent time lag. Unfortunately the magnitude of the frequency dependent delay predicted by this model is too small for our data to establish whether it is incorporated in a constant over-all time delay independent of frequency or whether it is added without interaction to a constant time delay. It is conceivable that this might be achieved by treating a very much larger body of data statistically.

SUMMARY

1. Accommodation measurements were obtained with a high-resolution, continuously recording infra-red optometer on six young emmetropic subjects when various focusing tasks were presented to them monocularly.

2. The reaction time of the accommodation responses to a focusing stimulus has an average value of 0·37 sec with a standard deviation of 0·08 sec.

3. Single-sweep responses are obtained during voluntary accommodation and also when a step focusing task is presented with a number of clues identifying the accommodation level required. These responses are to a first approximation exponential with a time constant of about 0·25 sec. Maximum velocities recorded during a 2 D response are about 10 D/sec.

4. The time which elapsed between onset of stimulus and the beginning of a reasonably steady accommodation level near the stimulus requirement is about 1 sec in the case of single-sweep responses. When the response has more than one phase, the time which elapsed may be 2 sec or more.

5. Responses to momentary optical defocusing and to simple harmonic defocusing follow each aspect of the stimulus after a reaction time. It is concluded that information about the state of focus may be assimilated during the act of accommodation and that accommodation movements can be modified during their course.

6. When the accommodation mechanism has to track the focus of a target, it appears to do so usually in steps, but the damping of the system and the superimposed fluctuations make the identification of the tracking strategy or strategies difficult.

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THE DISTRIBUTION OF 5-HYDROXYTRYPTAMINE, TETRAMETHYLAMMONIUM, HOMARINE, AND OTHER SUBSTANCES IN SEA ANEMONES

BY A. P. MATHIAS, D. M. ROSS AND M. SCHACHTER
From the Departments of Biochemistry, Zoology and Physiology, University College London

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Many years ago Richet (1902, 1905) demonstrated the presence of several toxic substances in extracts of tentacles of sea anemones, the study of which incidentally led to the discovery of anaphylaxis. Although several attempts have been made to identify these substances (Richet, 1905; Richet & Portier, 1936; Sonderhoff, 1936), none has been successful. Ackermann, Holtz & Reinwein (1923), however, identified tetramethylammonium in a sea anemone but did not determine its contribution to the toxic properties of anemone extracts.

The present experiments were undertaken in an attempt to identify thalassine, the pruritogenic substance in coelenterates (Richet, 1902, 1905) which is also a potent histamine liberator (Jaques & Schachter, 1954). We soon found, however, that a number of distinct pharmacologically active substances were present in sea anemones, and proceeded to identify them. We have, in fact, identified and determined the distribution of 5-hydroxytryptamine (5-HT), histamine, and tetramethylammonium (TMA); we have also found and studied the properties of another potent substance, probably a protein, which produces a marked and maintained contracture of the frog rectus abdominis muscle and a quick contraction, or twitch, of the sartorius muscle. Thalassine, however, is probably not identical with any of these.

In addition to the above pharmacologically active substances we identified the apparently inactive base, homarine (*N*-methyl picolinic acid betaine), in high concentrations in the tissues of all the sea anemones which we studied, and also in the Portuguese man-of-war, *Physalia*. Homarine, which we found in very high concentrations in some tissues, derived its name from the fact that it was discovered in the tissues of the lobster, *Homarus vulgaris* (Hoppe-Seyler, 1933).

METHODS

Animals. The sea anemones *Calliactis parasitica*, *Actinia equina*, *Anemonia sulcata* and *Metridium senile* were obtained from Plymouth, kept alive in tanks of sea water, and allowed to settle on glass plates. They were removed carefully to avoid closure of the animals and withdrawal of the tentacles. They were quickly frozen either by immersion in liquid nitrogen, or by covering them with powdered dry ice. Animals were dissected into various components—tentacle, outer body wall, and coelenteric structures. The latter unavoidably included some inner surface of the body wall. All tissues were kept frozen throughout dissection, as much frozen sea water as possible was discarded, and the dissected parts were freeze-dried. For the satisfactory separation of the thread-like acontia from the coelenteric tissues, eight specimens of *C. parasitica* were anaesthetized by placing them during the night in an aqueous solution of 7% magnesium chloride plus one half its volume of sea water.

A single living specimen of *Physalia* (Portuguese man-of-war) was obtained from Hastings, and freeze-dried tissues were prepared as described.

Isolated organ preparations. The isolated rat uterus and guinea-pig ileum were suspended in a 15 ml. bath in Tyrode solution and contractions were recorded by a frontal-writing lever on a smoked drum. The concentration of Ca^{2+} in the Tyrode solution was reduced to half the normal and the bath temperature was kept at 28–30° C (instead of 34° C) with the rat uterus. Atropine, mepyramine and lysergic acid diethylamide were added in concentrations of 10^{-4} g/l. when required. The isolated rectus abdominis muscle of the frog (*Rana temporaria*) was suspended in 8 ml. Ringer's solution, in some instances containing physostigmine (4×10^{-3} g/l.) and contractions were recorded on a smoked drum. Tubocurarine was added to a final concentration of 10^{-3} g/l. when required. The preparation was kept at room temperature. The isolated sartorius muscle of the frog was used in the same way.

The isolated perfused cat skin preparation was prepared as previously described (Feldberg & Paton, 1951; Feldberg & Schachter, 1952).

Extracts. For initial tests freeze-dried tissues were simply macerated in isotonic sodium chloride solution and centrifuged, and the supernatant solution tested. When testing quantitatively for histamine, 5-HT and TMA, the tissues were extracted with saline acidified to pH 3–4 with HCl, heated in a water-bath to 98° C for 1–2 min, cooled, centrifuged, neutralized and tested. In some instances extracts were made with 95% ethanol, taken to dryness *in vacuo*, redissolved in saline and tested, activity being expressed in reference to the original freeze-dried tissue.

For paper chromatography freeze-dried tissues were first extracted in a small volume of distilled water and centrifuged; the supernatant solution was removed, care being taken to leave the fatty layer behind, and dried *in vacuo*. The residue was then extracted three or four times with small volumes of 95% ethanol, centrifuged, and the alcohol extract dried. For chromatography, the latter dried residue was taken up in a small volume of water, centrifuged, the supernatant solution reduced in volume *in vacuo*, and applied to the paper.

Paper chromatography. Ascending chromatograms were run during the night at room temperature on Whatman No. 1 paper (except when otherwise stated). The following solvents were used: *n*-butanol–acetic acid–water (Partridge, 1948), *iso*propanol–hydrochloric acid (Lembeck, 1954), *n*-propanol–formic acid (Whittaker & Wijesundera, 1952), and *n*-butanol saturated with 0·2N ammonium hydroxide. Papers were cut into horizontal strips of 1·0 cm, eluted with saline or Tyrode solution (containing physostigmine, 4×10^{-3} g/l. when required) and tested on isolated muscle preparations. Extracts were prepared as described above and applied as a narrow strip on the paper. The extract applied to a 1 cm strip of paper was derived from 25–50 mg of original freeze-dried tissue.

Chromatograms were viewed under an ultra-violet lamp ('Chromatolete', Hanovia; λ, 254 mμ) and quenching or fluorescent areas marked out. They were developed with one

or more of the following reagents: Pauly's reagent (diazotized sulphanilic acid), ninhydrin–acetic acid (Jepson & Stevens, 1953), ninhydrin, chlorine–starch–iodide (Rydon & Smith, 1952), iodine spray (in 1% ethanol) or iodine vapour.

Preparation of homarine and other quaternary ammonium compounds

Homarine was prepared as described by Hoppe-Seyler (1933), trimethylammonium iodide by the method of Collie & Schryver (1890), and trimethylpropylammonium iodide by the method of Weiss (1892).

Ultra-violet absorption spectra. The areas on the chromatogram corresponding in R_F value or colour reactions with 5-HT, homarine, etc. were eluted in 2·5 ml. water, and the absorption spectra of the eluate determined in 1·0 cm quartz cells in the Hilger 'Uvispek' against a blank containing an aqueous extract of an equivalent area of the chromatogram corresponding in R_F value to the eluate. Hydrogen ion modifications for spectrophotometry were obtained by addition of small quantities of HCl or NaOH to blank and sample. Concentrations of homarine were calculated by using the molar extinction coefficient quoted by Keyl, Michaelson & Whittaker (1957).

Chemicals and reagents. Histamine and 5-HT were used as acid phosphate and creatinine sulphate, respectively, and values expressed as their bases.

RESULTS

5-*Hydroxytryptamine*

Very high concentrations (up to 600 μg/g freeze-dried tissue) of 5-HT were found in the soft tissues of the coelenteron of the anemone, *C. parasitica*. The concentrations were much less in the column (or body wall) and lowest in the tentacles (Table 1). The exact site of 5-HT in the coelenteric

TABLE 1. Distribution of 5-hydroxytryptamine in the sea anemone *Calliactis parasitica*

Preparation	No. of animals	Test	5-hydroxytryptamine (μg/g freeze-dried tissue)		
			Tentacle	Body wall (column)	Coelenteric tissues
I	5	Rat uterus	10	—	—
II	9	Rat uterus	12	30	—
II	9	Guinea-pig ileum	8	25	—
III	7	Rat uterus	6	36	600
IV	4	Rat uterus	7	15	500

tissues remains undetermined, but it cannot be in the fine thread-like structures present there, the acontia. These structures were pooled from approximately eight specimens of *C. parasitica* and contained only 20 μg/g freeze-dried tissue, whereas the remaining coelenteric tissue contained approximately 500 μg/g. Complete separation of the column from coelenteric tissue is not possible and perhaps the concentration of 5-HT found in the column is exaggerated by contamination with some internal structures.

The identity of the active substance in the extracts with 5-HT was established beyond doubt by a number of pharmacological and chemical

tests. It contracted the isolated guinea-pig ileum (in the presence of atropine and mepyramine) and the rat uterus, and its action on the latter was completely abolished by lysergic acid diethylamide (LSD). Alcohol extracts chromatographed on paper in either *n*-butanol–acetic acid or in *iso*propanol–hydrochloric acid yielded an eluate pharmacologically active when tested on the rat uterus. The area on the paper chromatogram from which this eluate was obtained gave the same colour reactions as 5-HT with ninhydrin, Ehrlich's reagent and Pauly's reagent. It also gave the green ultra-violet fluorescent reaction described by Jepson & Stevens (1953). This area, however, did not correspond exactly with the R_F value of the 5-HT marker strip when relatively impure extracts were chromatographed. Thus, in a typical chromatogram run in butanol–acetic acid, the pharmacologically active eluate had an R_F value of only 0·28, whereas that of a 5-HT marker was 0·40. Similarly, chromatography of the same extract in propanol–formic acid yielded an active eluate with an R_F value of 0·52, whereas that of 5-HT was 0·45 (Fig. 1). In both instances the area on the paper which yielded the pharmacologically active eluate gave the various colour and other reactions for 5-HT which were previously described. After rechromatography in *iso*propanol–hydrochloric acid, however, each of these eluates yielded an active material which now corresponded with synthetic 5-HT in R_F value as well as in the other reactions (Fig. 2). In addition, these purified, rechromatographed eluates yielded ultra-violet absorption curves which closely agreed with those of 5-HT at a pH of 5·6 and also when the curve was determined at pH 12 after addition of 2N-NaOH.

In contrast to the high concentration of 5-HT which we found in *C. parasitica*, we failed to detect it in any of the tissues of *M. senile* or *A. sulcata*. Extracts from these species contained either no 5-HT or certainly less than 2·0 μg/g freeze-dried tissue. We were unable also to detect 5-HT on paper chromatograms of alcohol extracts of these species. The freeze-dried tissues of *Physalia* also contained less than 2·0 μg/g. Extracts of *A. equina* possessed some activity resembling that of 5-HT on the rat uterus, but the low concentrations, approximately 5 μg/g freeze-dried tissue, did not permit a definite identification with 5-HT. Our failure to detect 5-HT in the tissues of *M. senile* or of *Physalia* is in contrast to Welsh's (1956) findings of high concentrations in the tentacles of these animals. Despite the absence of 5-HT, *Physalia* tentacle was extremely toxic. The intraperitoneal injection of freeze-dried tentacle (50 mg/kg) in rats and guinea-pigs caused death within 24 hr with signs of severe pulmonary oedema.

Unheated extracts of *A. equina* and *A. sulcata* contained another substance which also contracted the isolated rat uterus. The delay preceding

contraction in this instance was slightly longer than with 5-HT, the uterus subsequently relaxed more slowly and also tended to become desensitized with repeated tests. The concentration of this substance, expressed as 5-HT equivalent activity, was 20–40 μg/g in *A. equina* and approximately 10 μg/g in *A. sulcata*. It was inactivated by heating to 98° C for 2 min and was non-dialysable.

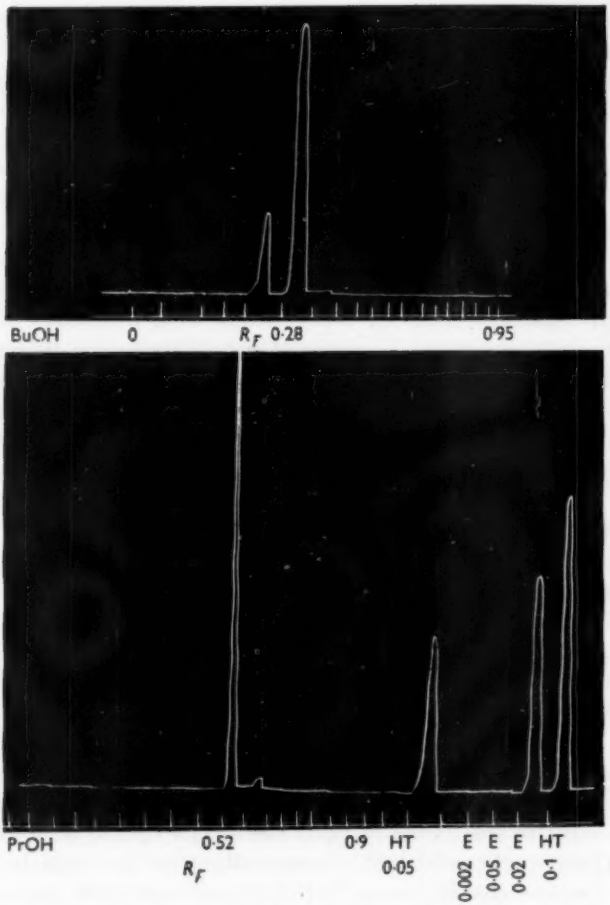


Fig. 1. Contractions of isolated rat uterus (atropine and LSD, 10^{-4} g/l.) to eluates from chromatograms in two different solvents of alcohol extract of the sea anemone, *A. equina*. BuOH, eluates from chromatogram in *n*-butanol–acetic acid solvent. PrOH, eluates from chromatogram in *iso*propanol–hydrochloric acid solvent. HT, 5-HT creatinine sulphate, μg. E, active eluate, ml.

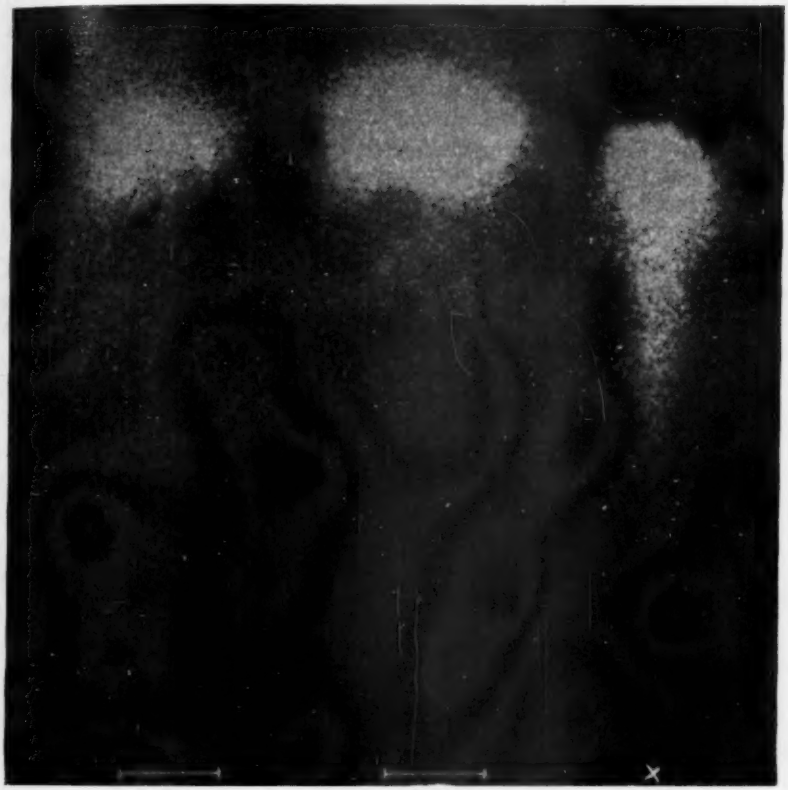


Fig. 2. Rechromatographed extract from *C. parasitica* coelenteric tissue. Ultraviolet light photograph. Solvent, *iso*propanol–hydrochloric acid. BuOH, active eluate from previous chromatogram in *n*-butanol–acetic acid solvent (Fig. 1). PrOH, active eluate from previous chromatogram in *iso*propanol–hydrochloric acid (Fig. 1). 5-HT; marker of 5-HT creatinine sulphate.

Histamine

Histamine was found in the freeze-dried tissues of two of the four species of anemones studied, *A. equina* and *A. sulcata*. It was not detected in the tissues of *C. parasitica*, *M. senile* or in *Physalia* (< 2 μg/g freeze-dried tissue). When histamine was present the highest concentration was in the tentacles (Table 2).

The activity of these extracts was indistinguishable from that of histamine on the guinea-pig ileum, and was antagonized by mepyramine. Furthermore, the extracts of tentacles of *A. equina* chromatographed on

paper in *n*-propanol–formic acid showed a positive Pauly reaction which, on elution, was pharmacologically identical with that of histamine. The high concentrations of 5-HT in the coelenteric tissues of *C. parasitica* made it difficult to eliminate the possible presence of minute amounts of histamine. However, when large amounts of extract of this tissue were tested, the response was completely unaffected by mepyramine, which suggests that histamine was absent.

TABLE 2. Distribution of histamine in various sea anemones

Species	No. of animals	Histamine (μg/g freeze-dried tissue)		
		Tentacle	Body wall (column)	Coelenteric tissues
A. equina I	4	150	*125	—
II	10	50	*35	—
A. sulcata I	2	25	—	—
II	8	20	5	—
III	4	20	8	10
C. parasitica I	5	2	2	2
II	7	2	2	2
M. senile II	4	2	2	2

* In this species a satisfactory separation of body wall (column) from coelenteric tissues was impossible. These values (body wall) are those of the whole animal minus tentacles.

Tetramethylammonium

Saline (0·9 %, w/v) extracts of *A. equina* and *A. sulcata* (but not of *C. parasitica* or *M. senile*) produced a strong contracture of the frog rectus abdominis muscle. Similar extracts of *Physalis* also caused contracture but they were much less effective.

It soon became evident that these extracts of *A. equina* contained two substances, chemically very different, both of which contracted the rectus muscle. One of these was dialysable and soluble in 95 % ethanol, the other non-dialysable and insoluble in ethanol. Thus, following the dialysis of saline extracts of *A. equina* (50–100 mg freeze-dried tentacle in 2 ml. against 8 ml. Ringer's solution for 2 hr) the dialysate produced a contracture of the rectus muscle which resembled that caused by acetylcholine in rate of onset and in subsequent relaxation, whereas the residue produced an irregular and long-maintained contracture. The amount of non-dialysable activity is far greater than that of the dialysable, although the two cannot be compared precisely because of the different types of contracture which they cause.

Alcohol (95 % ethanol) extracts of *A. equina* contained the dialysable substance only. It produced an acetylcholine-like contracture which was completely antagonized by tubocurarine (10^{-3} g/l.) (Fig. 3*a*). It was stable

on heating to 98° C for 5 min in N/100-HCl. Unlike esters of choline it was also stable on heating for 5 min in N/100-NaOH and was unaffected by incubation with horse plasma (1:1 (v/v)) at 37° C for 20 min. It was absorbed by the ion-exchange resin IRC-50 (in the H^+ form) from which it was released by acid. These properties suggested that the alcohol-soluble substance was a simple alkyl quaternary ammonium compound. In subsequent chromatographic experiments on paper it was, in fact, indistinguishable from tetramethylammonium (TMA). The freeze-dried tentacles from different batches of *A. equina* contained 1500–2000 μg/g in terms of TMA equivalent activity in alcohol extracts tested on the frog rectus preparation. The remainder of *A. equina* (minus tentacle) contained approximately one half the concentration of this substance.

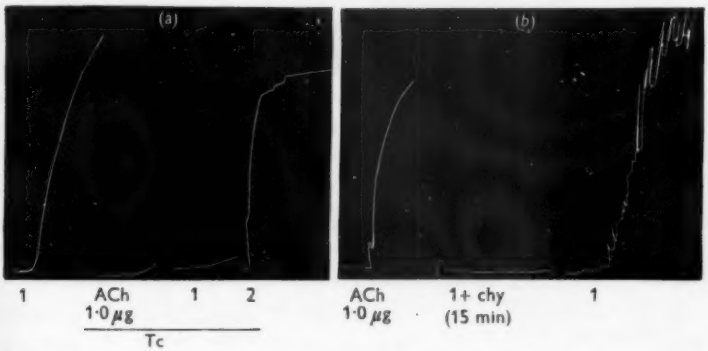


Fig. 3. Response of frog rectus abdominis muscle to extracts of freeze-dried tentacle from sea anemones *A. equina* and *A. sulcata*. ((*a*) and (*b*) are different rectus preparations.) (*a*) Tubocurarine, 10^{-3} g/l. (Tc), abolishes the contracture produced by the alcohol-soluble substance in *A. equina* but effect of saline extract persists. 1, alcohol-soluble material from 50 mg tentacle; 2, saline extract from 2 mg tentacle. (*b*) Saline extract of *A. sulcata* produces contracture with superimposed quick contractions. Effect abolished by incubation of extract with chymotrypsin. 1, saline extract from 5 mg tentacle (0·5 ml.); chy, 50 μg crystalline chymotrypsin (0·5 ml.).

Alcohol extracts of *A. sulcata* had one-tenth or less the activity of *A. equina* in contracting the frog rectus. The low concentrations of this substance in *A. sulcata* did not permit its identification. Alcohol extracts from large amounts of tissue of *C. parasitica*, *M. senile* and *Physalia* failed to cause contracture of the rectus muscle. It is very unlikely, therefore, that these tissues contain significant amounts of pharmacologically active esters of choline or active alkyl quaternary ammonium compounds. These findings differ from those of Welsh (1955), who obtained

evidence of high concentrations of murexine (urocanylcholine) in the tentacles of *Metridium* and *Physalia*.

Alcohol extracts of tentacle or body of *A. equina* chromatographed on paper in *n*-propanol–formic acid yielded an eluate which caused contracture of the frog rectus muscle (Fig. 4), reacted with iodine, and corresponded closely in R_F with TMA iodide. By rechromatographing such an active eluate in two solvents (*n*-propanol–formic acid or *n*-butanol–acetic acid),

Fig. 4. Contractions of frog rectus abdominis muscle to eluates from paper chromatogram of extract of *A. equina*. 0 = strip 1 cm either side of origin; 2–10, strips 2 cm wide.

TABLE 3. R_F values and other properties of rechromatographed active eluate from extract of *Actinia equina* and various quaternary ammonium compounds in two solvents

Solvents	*n*-propanol–formic acid		*n*-butanol–acetic acid	
Tests	Ultra-violet	I_2 reaction	Ultra-violet	I_2 reaction
TMA	—	0·48 grey	—	0·25 grey
TMEA	—	0·57 orange	—	0·32 orange
TMPA	—	0·70 orange	—	0·40 orange
ACh	—	0·58 orange	—	0·32 orange
Murexine	0·15 *Q*	0·15 orange	0·12 *Q*	0·12 orange
Eluate	0·17 *F*	—	0·25 *Q**	0·25 grey
	0·27 *Q*	—	0·32 *F*	—
	0·39 *F*	0·39 orange	0·40 *F*	—
	—	0·48 grey	0·49 *F*	—

TMA, TMEA and TMPA, tetramethyl-, trimethylethyl-, and trimethylpropylammonium iodide. *Q* and *F*, quenching and fluorescent reactions, respectively, in ultra-violet light. [ ] Area of eluate contracting frog rectus muscle.

* In this solvent an ultra-violet-quenching material did not clearly separate from the active substance.

pharmacological activity again appeared in both solvent systems at an R_F identical with that of TMA and reacting with iodine. It was readily distinguished chromatographically from ACh, urocanylcholine, and other quaternary ammonium compounds. The results of such an experiment are shown in Table 3.

The possibility that low concentrations of TMA exist in *M. senile*, *C. parasitica* or *Physalia* cannot be excluded, since chromatograms of their tissues show a number of iodine-reactive bands. However, since these bands when eluted do not contract the rectus muscle, and since extracts of large amounts of tissue are inactive, the concentration of active esters of choline or of tetramethylammonium must be very small.

Unidentified protein-like substance

Saline extracts of 1–10 mg freeze-dried tentacle of *A. equina* or *A. sulcata* produced a maximal, maintained contracture of the frog rectus abdominis muscle. This contracture differs from that produced by ACh or TMA in that frequently there are superimposed quick contractions (Fig. 3*b*). The contracture is also protracted, and with larger doses was apparently irreversible, the muscle failing to relax even after 90 min or longer; even with smaller doses it was usually maintained for 40 min or longer. This property made it difficult to study repeated effects on the same muscle preparation. In some instances the muscle showed repetitive quick contractions, resembling simple twitches, even after the extract was washed out. Both the contracture and quick contractions of the rectus muscle were unaffected by concentrations of tubocurarine (10^{-3} g/l.) which blocked the action of ACh, TMA, or the alcohol extract of *A. equina* (Fig. 3*a*). The effectiveness of this substance is apparent by the fact that the freeze-dried tentacle of *A. equina* contains an ACh equivalent activity of approximately 500 μg/g when tested on the frog rectus muscle. The tentacles of *A. equina* contain about twice the activity as the rest of the organism as was also found in the case of TMA. The tissues of *A. sulcata* contain approximately one third the activity of *A. equina*.

At similar concentrations to those producing contracture of the rectus abdominis muscle, this substance, like ACh, produced a quick contraction, or twitch, of the isolated frog sartorius muscle. The effect still occurred in the presence of tubocurarine (10^{-3} g/l.) when the response to ACh was completely blocked. This muscle, like the rectus, also showed repetitive, quick contractions during, or after, exposure to the extract.

The substance failed to dialyse across a cellophane membrane (24 hr, 4° C) and was insoluble in 95 % ethanol, the insoluble residue being fully active when taken up in water. It was completely inactivated by heating for 5 min in a boiling water-bath in N/100-NaOH and also on incubation with crystalline chymotrypsin (500 μg/ml., 37° C, 20 min) (Fig. 3*b*). It was relatively stable, however, on heating in N/100-HCl.

Homarine (N-methyl picolinic acid betaine)

We were impressed early in our experiments by an ultra-violet-absorbing band in all our chromatograms. This band also reacted with iodine. Definite evidence that this substance was homarine was obtained by paper chromatography and by measurement of its ultra-violet absorption spectrum.

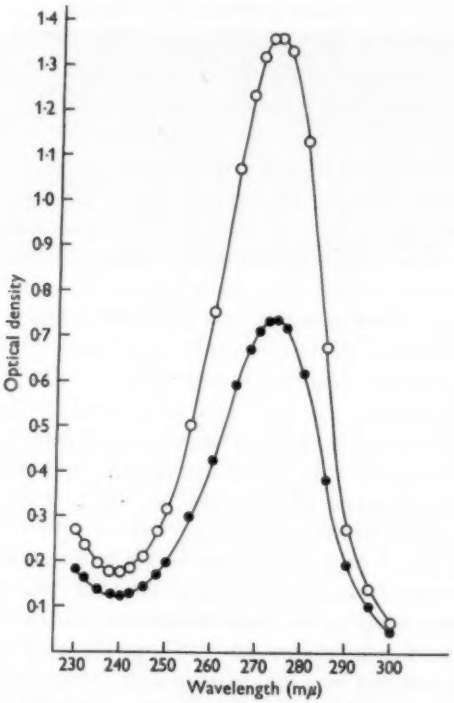


Fig. 5. Ultra-violet absorption spectra of synthetic homarine and eluate corresponding to homarine in R_F and other properties, in chromatogram of *A. equina* (body). *n*-Propanol–formic acid solvent. Eluate pH, 5.

Extracts of the anemones and of *Physalia* chromatographed in *n*-propanol–formic acid showed a strong ultra-violet-quenching, iodine-reactive area, with an R_F value identical with that of synthetic homarine. The eluate from this band on the paper in each case showed the identical ultra-violet absorption maximum and minimum values of synthetic homarine, at 273 and 240 mμ, respectively. As with homarine there was no shift in these values when the pH was adjusted with HCl or NaOH to pH 2 or 12. The concentrations of homarine were calculated by using the

molar extinction coefficient quoted by Keyl *et al.* (1957) and are shown in Table 4. They are, in general, very high, particularly in the tentacles of *A. equina* and in the coelenteric tissue of *C. parasitica*, namely 1·5 and 2·1 mg/g freeze-dried tissue, respectively.

The identification of homarine was confirmed by further procedures in the case of extracts of *A. equina*. In this instance, extracts of body (whole minus tentacle) were chromatographed on Whatman No. 4 paper in three different solvents, with synthetic homarine as a marker. R_F values of 0·36, 0·31, and 0·09 were obtained for synthetic homarine in *n*-propanol–formic acid, *n*-butanol–acetic acid, and *n*-butanol–ammonium hydroxide solvents. A band with identical R_F values, also reacting with iodine and showing strong ultra-violet-quenching properties, was obtained with the extract in all three solvents. On elution, these bands were found to have ultra-violet absorption spectra identical with that of homarine (Fig. 5); as with homarine they were unaffected by altering the pH of the solution to 2 or to 12.

Other substances

Chromatograms in propanol–formic acid of extracts of all these coelenterate tissues regularly showed a marked iodine-reacting band with a low R_F value of approximately 0·2. This area was not visible when exposed to ultra-violet light, did not react with Pauly's reagent, and the eluate was inactive on the frog rectus muscle. An apparent positive ninhydrin reaction proved to be negative when the iodine-reacting area was eluted and rechromatographed in *n*-butanol–acetic acid. In this solvent the ninhydrin-positive area separated clearly from the one reacting with iodine. Hydrolysis of the eluate (in N/10-NaOH, 98° C, 5 min) did not alter the R_F of the iodine-reacting band. This substance, therefore, is not a choline ester. It was also distinguished from betaine, which had a much higher R_F value, approximately 0·38 in the same solvent, and similarly from simple alkyl quaternary ammonium compounds which had relatively high R_F values (Table 3). It was also distinguished from γ-aminobutyric acid which had an R_F value of 0·45 in this solvent, and which, as expected, gave a strong ninhydrin reaction. This substance remains, as yet, unidentified. We have not excluded the possibility that it is γ-butyrobetaine which was identified by Ackermann (1927) in the tissues of *A. equina*.

DISCUSSION

Three of the four pharmacologically active substances found in the coelenterates studied are present in the highest concentrations in the tentacles. They are histamine, tetramethylammonium, and a toxic protein-like substance. It would seem, therefore, that they are related to

the presence of nematocysts, but their presence in relatively high concentrations in other parts of the animals indicates that they are not likely to be associated solely with these stinging cells.

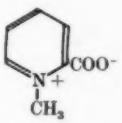
The major toxic component appears to be the non-dialysable substance, probably a protein, which produces a relatively irreversible contracture of the rectus abdominis and a twitch of the sartorius muscle of the frog. The nature of the response of the rectus muscle to this substance suggests that it activates both the slow (contracture) and quick (twitch) fibres under conditions in which cholinesters and alkyl quaternary ammonium compounds activate only the slow fibres. The fact that, like acetylcholine, it produces a twitch of the isolated sartorius muscle is consistent with this possibility, since the sartorius muscle of the frog is thought to contain fibres of the 'quick' type only (Kuffler, 1953, 1955). The remarkable activity of this substance on frog skeletal muscle suggests that an analysis of its action may be of interest. Toxic proteins with marked effects on skeletal muscle have been found recently in the salivary gland of the octopus and cuttlefish (Ghiretti, 1959) and in bee venom (Neumann & Habermann, 1954). There may be a group of such toxic proteins with an action on skeletal muscle.

There is a difference in pharmacological activity of extracts from the different anemones. Thus, whereas extracts from *A. equina* are extremely toxic, those from *M. senile* are pharmacologically inert. This difference would appear to correlate with their habits, in so far as *A. equina* is a sessile but predatory organism whereas *M. senile*, with its dense fine tentacles, is probably a passive 'filter-feeder'. Similarly, *C. parasitica*, which lives on the shell of the hermit crab, is believed to share food already captured by the crab (Orton, 1922; Stephenson, 1935).

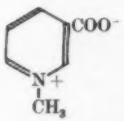
The high concentration of 5-HT in the coelenteric tissues of *C. parasitica* is in contrast with its absence in other anemones. A similar situation in regard to 5-HT exists in Octopoda, where the salivary gland of *O. vulgaris* contains approximately 500 μg/g whereas that of *O. macropus* contains none (Erspamer, 1954). Our finding that the lowest concentration of 5-HT in *C. parasitica* is present in the tentacles does not support the suggestion of Welsh (1955) that this substance is a specific nematocyst toxin in *Metridium* and in *Physalia*. Indeed, we could find no evidence for the presence of significant amounts of 5-HT in any of the tissues of these two animals. This does not support the view (Editorial, *Brit. med. J.* 1957) that the toxic reaction in man following contact with *Physalia* tentacle is in part due to the action of 5-HT.

Our results demonstrate the widespread distribution and high concentration of homarine (*N*-methyl picolinic acid betaine) (Fig. 6) in coelenterate tissues. Little has been learned about this base since it was dis-

covered by Hoppe-Seyler (1933) in the tissues of the lobster *Homarus vulgaris*, the lamellibranch *Arca noa*, and the sea urchin *Arbacia pustulosa*. Recently, however, Ackermann (1953) has identified the closely related base trigonelline (*N*-methyl nicotinic acid betaine) (Fig. 6) together with homarine in extracts of the sea anemone, *A. sulcata*, and Gasteiger (Gasteiger, Gergen & Haake, 1955) has drawn attention to the high concentrations of homarine in marine arthropods and molluscs. Homarine has no known pharmacological activity, and as yet there are no reports of its presence in non-marine or vertebrate animals. It would seem likely that a substance occurring in such high concentrations in several large invertebrate phyla is of biological significance, and it would be of interest to extend the study of its distribution in nature.



Homarine
(*N*-methyl picolinic acid betaine)



Trigonelline
(*N*-methyl nicotinic acid betaine)

Fig. 6. Structure of homarine and trigonelline.

The chemical nature of thalassine, which is the pruritogen and histamine liberator in anemone extracts, has not been solved. It has been claimed that thalassine is, in fact, tetramethylammonium (Courville, Halstead & Hessel, 1958). This cannot be so, however, since we administered varying doses of TMA to dogs, subcutaneously or intravenously, and failed to produce any sign of the syndrome described by Richet (1902). Also, TMA failed to release histamine from perfused cat skin preparations. The substance we described, with an action on skeletal muscle like Richet's congestin which produces pulmonary oedema, is probably a protein, but it remains to be determined whether they are the same or different substances.

SUMMARY

1. The identification and distribution of the pharmacologically active substances, 5-hydroxytryptamine, histamine and tetramethylammonium, were determined in four species of sea anemones and in the Portuguese man-of-war, *Physalia*, by pharmacological and chemical tests. In those species where they were identified, histamine (*Actinia equina* and *Anemonia sulcata*) and tetramethylammonium (*A. equina*) were present in the highest concentrations in the tentacles. 5-Hydroxytryptamine, however, was present in very high concentrations in the coelenteric tissues of one

species (*Calliactis parasitica*), and in much lower concentrations in the tentacles.

2. A substance, probably a protein, with an action on skeletal muscle, was present in tentacles, and to a less extent in other tissues, of *A. equina* and *A. sulcata*. It produced a maintained contracture of the frog rectus muscle with superimposed quick contractions, and a quick (twitch) contraction of the frog sartorius muscle. The high concentration of this substance and its potency suggest that it is the major toxic agent in the tentacles of these anemones.

3. Homarine (*N*-methyl picolinic acid betaine) was identified in high concentrations in the different parts of all the animals examined.

We gratefully acknowledge a generous supply of synthetic murexine hydrochloride from Dr V. Erspamer.

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THE ACUTE EFFECT OF HYDROCORTISONE, DEOXYCORTICOSTERONE AND ALDOSTERONE UPON THE EXCRETION OF SODIUM, POTASSIUM AND ACID BY THE HUMAN KIDNEY

BY J. N. MILLS, S. THOMAS AND K. S. WILLIAMSON
From the Department of Physiology, University of Manchester

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It is now generally agreed that an important action of adrenal steroids is to stimulate the reabsorption of sodium by the renal tubules. The mechanism is more debatable; but it is often tacitly assumed that the mineralocorticoid action of different steroids is the same, if the dose is suitably adjusted.

The direct renal effects are best studied in acute experiments extending over a few hours, as secondary effects, due to disturbance of total body supplies of electrolytes, are thus minimized. Since cation excretion varies markedly at different times of day, any effect of steroids upon renal cation excretion must be assessed by comparison with control experiments, in order to take account of this diurnal rhythm.

In a series of observations in the early afternoon, which have already been briefly reported (Mills, Thomas & Williamson, 1959*a*), it appeared that hydrocortisone (cortisol) promoted tubular reabsorption of sodium in exchange for potassium, without any increase in output of hydrion, whereas aldosterone and deoxycorticosterone (DOC) increased the excretion of both hydrion and potassium in exchange for reabsorbed sodium. These observations have now been extended, by measuring the effect of these steroids under three other conditions: in the morning, when excretion of potassium is normally high and of acid low; at night, when the reverse condition prevails (briefly reported by Mills, Thomas & Williamson, 1959*b*); and in the afternoon after administration of acetazoleamide, which by inhibiting carbonic anhydrase blocks a large fraction of renal tubular acid secretion.

METHODS

Plan of experiments. The subjects were healthy males aged 21–44. Where subjects have participated in our earlier experiments (Longson, Mills, Thomas & Yates, 1956; Mills & Thomas, 1957, 1958) the same initial letters are used to refer to them. Four series of experiments have been performed: afternoon, morning, night and afternoon with acetazoleamide. Each subject participating in any series performed at least one control experi-

ment without injection; circumstances—timing, posture, etc—were closely similar in all experiments of one series upon any subject. Except at night, the subject usually remained seated, apart from rising to void urine. A light breakfast was eaten on the morning of an experiment, but no food thereafter. No caffeine was consumed, nor was it for 4 hr before bedtime in night experiments. Subjects drank freely.

Steroids used have been hydrocortisone (free alcohol) (Upjohn), 100 mg, supplied dissolved in 20 ml. 50% ethyl alcohol, and added to 0·5 l. isotonic lactose before intravenous injection; hydrocortisone hemisuccinate (Upjohn or Glaxo), 133·7 mg, representing 100 mg hydrocortisone, dissolved in 2–4 ml. water and given intravenously; deoxycorticosterone glucoside (DOCG) (Ciba), 20 or 30 mg given intravenously; DL-aldosterone (Ciba) 0·5 mg, either the 21-monoacetate in 95% ethanol, diluted with isotonic NaCl, or the free alcohol in aqueous solution. DOCG was used in earlier experiments, before aldosterone was available. Its action appears identical with that of aldosterone and its use was discontinued. Where mean results of aldosterone injection have been calculated, experiments with DOCG have been included with them.

In afternoon experiments, subjects sat down usually at 11.00 hr, and received injections at about 13.00 hr. The dose of aldosterone was given to F and T in a single injection, and to A, K and M in two equal portions separated by 1½ hr. Urine was usually voided every half hour, except in control experiments, when it was voided hourly. A few of these experiments have already been briefly described (Mills & Thomas, 1958).

Morning experiments were similar, except that the injection was given about 9.30, when the subject arrived and sat down in the laboratory.

In night experiments the whole urine produced during a night's sleep of about 8 hr was collected. Injections were given immediately before going to bed, and in most experiments aldosterone was given intramuscularly to prolong its action. In a few further experiments the night urine was collected in two consecutive portions.

Acetazoleamide experiments were similar to the afternoon series, but in addition 250 mg acetazoleamide (Diamox, Lederle) was given, orally at 11.00 hr to M and intravenously at 12.30 hr to A and W. Rapidity of absorption after oral administration seems to vary between individuals: alkalinity of the urine indicated that M absorbed the drug rapidly, whilst when W took it by mouth the rise of urinary pH was inconveniently slow. Steroids, when given, were always injected at 12.30 hr.

Analytical methods. The following procedures were used: pH, glass electrode at 37° C; titratable acid, potentiometric titration with N/5-NaOH to pH 7·4 at 37° C; ammonium, aeration and titration (Hawk, Oser & Summerson, 1947); total CO_2, Van Slyke & Neill (1924) bicarbonate was calculated from total CO_2, pH and the pK_1 of $6{\cdot}32-0{\cdot}5\sqrt{B}$ (Hastings & Sendroy, 1925); B was taken as $[Na+K+NH_4]$; these analyses were almost always completed on the day of the experiment; sodium and potassium, flame photometer, EEL (Collins & Polkinhorne, 1952); inorganic phosphate, Fiske & Subbarow (1925); creatinine, Bonsnes & Taussky (1945).

Hydrion output (H) was calculated as ammonium + titratable acid − bicarbonate. In many of the earlier experiments titratable acid was not determined but that fraction due to phosphate was calculated (Longson & Mills, 1953). Hydrion thus calculated is designated H′.

In considering cation pattern in urine, ammonium + titratable acid is referred to as h, ammonium + $H_2PO_4^-$ as h′, and $Na+K+h$ or h′ as B.

RESULTS

Afternoon experiments

Complete series of experiments, including at least one control and one each with injection of cortisol, aldosterone, and DOCG, were performed on

subjects A, F, K, M, and T. In the control experiments, when no injection was given, progressive changes were observed in electrolyte excretion between 10.30 and 17.00 hr. Potassium excretion (Fig. 1) always declined, as did sodium excretion (Fig. 2) in all subjects except F, in whom it was low at the start. Na:K ratio (Fig. 2) always rose, although in M the rise was slight. Sodium and hydrion always formed an increasing, and potassium

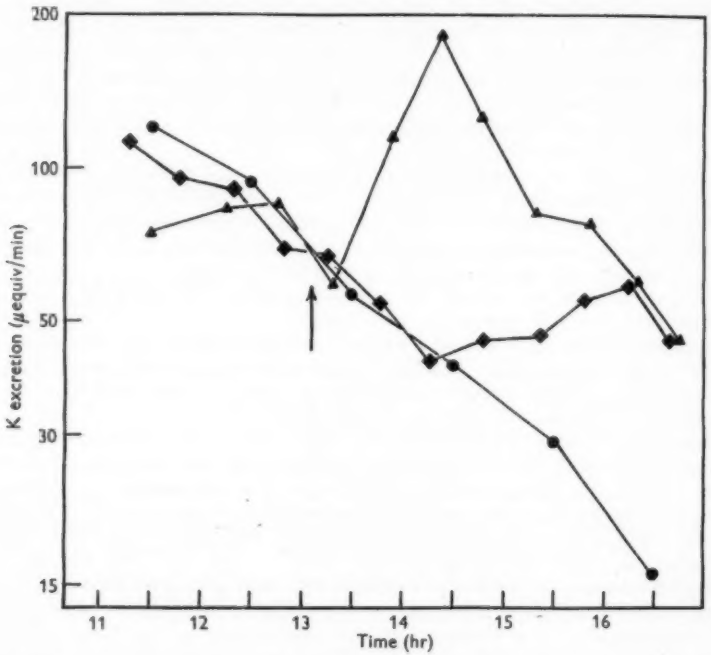

Fig. 1. Afternoon expts., subject T. Potassium output in urine, log. scale: ●, control. ◆, aldosterone. ▲, cortisol. Arrow indicates time of steroid injection. In this, as in all other plots of urinary variables against time, the time is the mid point of the period of collection.

a decreasing, proportion of the total urinary cations. After every steroid injection, in every subject, sodium excretion fell further than in control experiments, and the Na:K ratio (Fig. 2), instead of rising, fell sharply. Potassium excretion, both absolute (Fig. 1) and as a proportion of total cations, rose after cortisol injection; after aldosterone or DOCG K:*B* remained unaltered or rose, but only in subject M was the rise as great as after cortisol.

Differences in hydrion excretion manifest themselves as differences in output of ammonium, titratable acid and bicarbonate, and in urinary pH.

The effects of the various steroids upon urinary pH, which is closely correlated with bicarbonate concentration, are shown in Table 1. The period between 2 and 2½ hr after steroid injection has been chosen to assess the differences, as at this period all the steroids seem to be active. It will be seen that in all subjects except M the pH after cortisol was higher than in any other experiment. In this subject the action of cortisol was slow, and higher values of pH were observed later.

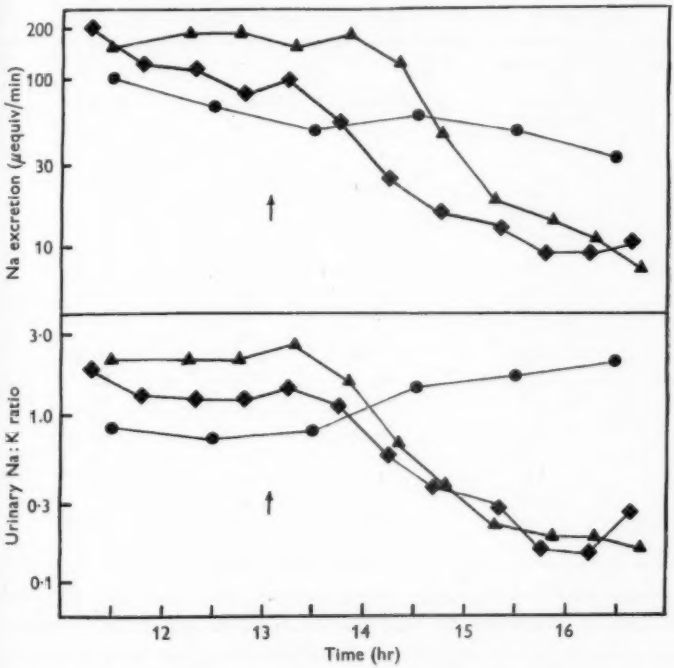

Fig. 2. Afternoon expts., subject T. Sodium output, and Na:K ratio in urine, log. scale. Symbols as in Fig. 1.

The effect of cortisol on pH is even clearer if one considers the change in pH after injection, since in the afternoon urinary pH usually fell steadily and more so after aldosterone and DOCG. After cortisol pH always rose, and only in one experiment with aldosterone was a comparable rise seen. This pH change will be discussed further when changes in phosphate excretion are described.

Owing to considerable changes in total cation excretion, hydrion outputs are best considered as a proportion of the total cations. This proportion $h' : B$, is shown in Table 1 for the period 2–2½ hr after steroid

administration. After aldosterone it was usually increased but sometimes unaltered: after cortisol it was unaltered or reduced. By contrast K:*B* was highest after cortisol, and Na:*B* was very low after all steroids.

There thus appears to be a difference between the action of cortisol and that of aldosterone, in that cortisol seems to promote sodium reabsorption in exchange for secreted potassium, with perhaps a drop in hydrion secretion, whilst aldosterone promotes sodium reabsorption in exchange for an

TABLE 1. pH of urine, and outputs of Na, K and h′ expressed as a percentage of total cations, 2–2½ hr after injection of hormone, or at a corresponding time for control expts. Afternoon expts.

Subject		Control	Aldosterone	Cortisol
A	Na	47	11	19
	K	31	52	71
	h′	22	37	10
	pH	6·24	6·11	6·47
F	Na	62 48	30	31
	K	23 19	53	64
	h′	15 33	17	5
	pH	5·64 5·85	5·92	6·83
K	Na	60	22	27
	K	22	32	52
	h′	18	46	21
	pH	5·52	5·21	6·08
M	Na	48	14	21
	K	25	37	47
	h′	27	49	32
	pH	6·30	5·95	5·84
T	Na	45	16	16
	K	31	57	70
	h′	24	27	14
	pH	6·15	5·48	6·60

increased secretion of both potassium and hydrion The urinary h′:*K* ratio discriminates sharply between the action of these steroids. This ratio rose in all control experiments and all those after injection of aldosterone. Cortisol always reduced or even reversed this change. At midday potassium excretion was as much as ten times as high as hydrion excretion, whilst by 16.00 hr hydrion excretion roughly equalled that of potassium except when cortisol had been injected. The h′:*K* ratios at 12.00 and at 16.00 are shown in Table 2, and the full time course of h′:K for experiments on subject T in Fig. 3. Despite the fact that the times chosen were not in every experiment optimal for demonstrating the action of the hormones, and that more convincing figures could be presented by choosing a time apparently optimal for each experiment, the difference between the actions of the steroids is clear. It appears that in the experiment of Fig. 3 this effect of cortisol was wearing off in the last periods, covering the fourth hour after injection.

It has already been briefly reported (Mills & Thomas, 1958) that phosphate excretion falls after cortisol injection, probably as a result of the fall

of plasma concentration (Mills & Thomas, 1957, 1959). The same fall in phosphate excretion has been observed in the present experiments, as is seen in Fig. 4. All subjects at some stage after cortisol injection were excreting under 10 μmole/min of phosphate. The timing of this fall was somewhat variable in different subjects, but was roughly synchronized with the rise of pH which has already been mentioned. In subjects K and M both changes were slow and pH in later periods rose beyond the values recorded for cortisol in Table 1.

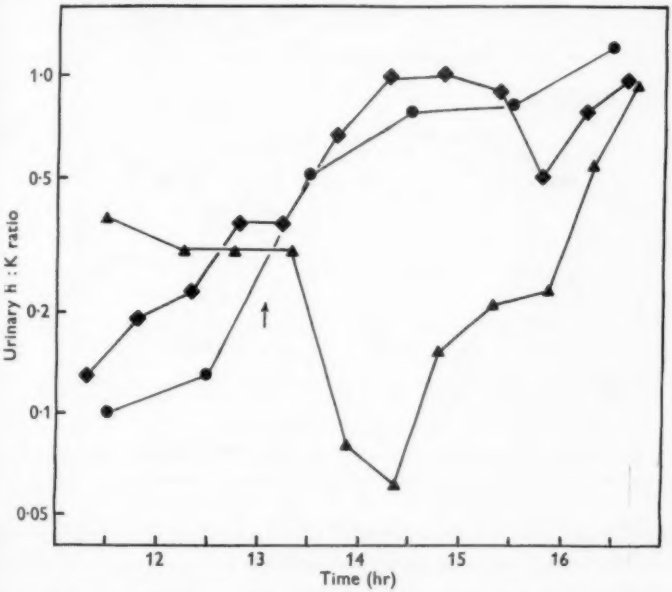

Fig. 3. Afternoon expts., subject T. Urinary h′:K ratio, log. scale. Symbols as in Fig. 1.

TABLE 2. Urinary hydrion:potassium ratio (h′:K) at 12.00 and at 16.00 hr

Subject	Control	Aldosterone	Cortisol
A 12·00	0·27	0·46	0·24
16·00	1·32	0·88	0·24
F 12·00	0·15 0·23	0·12	0·15
16·00	0·76 1·30	0·35	0·11
K 12·00	0·36	0·30	0·23
16·00	2·39	2·12	0·41
M 12·00	0·13	0·16	0·22
16·00	1·08	0·87	0·47
T 12·00	0·10	0·19	0·38
16·00	1·20	0·50	0·23

Cortisol or DOCG has been injected into four further subjects upon whom control observations have not been made. The results support our previous conclusions. In particular, potassium excretion has always risen after cortisol injection, at a time of day when a falling excretion is regularly observed under control conditions.

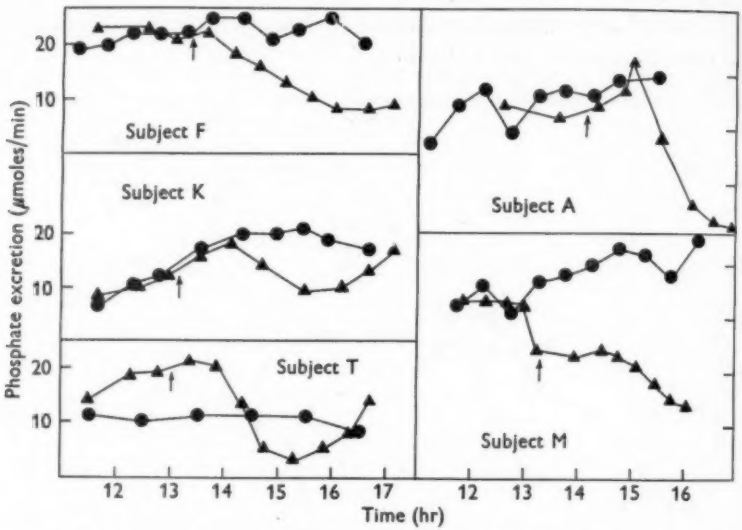

Fig. 4. Afternoon expts., subjects F, K, T, A and M. Urinary phosphate output in control expts. and after cortisol injection. Symbols as in Fig. 1.

Creatinine excretion was measured in all experiments, both as a check upon bladder emptying and as an indication of any changes in glomerular filtration rate (G.F.R.). The mean output before injection varied between 1·05 and 1·36 mg/min in different subjects. The mean changes in each successive hour after steroid injection are shown in Fig. 5. These were barely significant, but they suggest a small rise after cortisol, and perhaps a small fall after aldosterone and DOCG.

Acetazoleamide experiments

Acetazoleamide, in inhibiting carbonic anhydrase, drastically reduces (Hanley, Jowett, Kilpatrick & Platts, 1959) or even abolishes (Schwartz & Relman, 1954) the renal tubular secretion of hydrion. A series of control cortisol and aldosterone experiments was therefore performed upon subjects A, M and W under the influence of acetazoleamide. In these experiments, urinary pH usually rose above 7·4, and ammonium excretion fell to a very low level of 2–14 μequiv/min and remained there throughout

all experiments, whether or not hormones were injected. Bicarbonate excretion rose to a high level of 300–700 μequiv/min, which is nevertheless far below the likely filtered load. Thereafter it fell steadily, reaching levels of 50–300 μequiv/min 4 hr after injection; the peak level of bicarbonate output was usually in the first half hour after intravenous injection, or in the second half hour after oral administration to subject M. It is uncertain how far the falling excretion of bicarbonate reflects a wearing off of the inhibition of carbonic anhydrase, or a diminishing filtered load. Since we measured neither plasma bicarbonate concentration nor glomerular filtration rate, it is impossible to calculate the rate of tubular reabsorption of

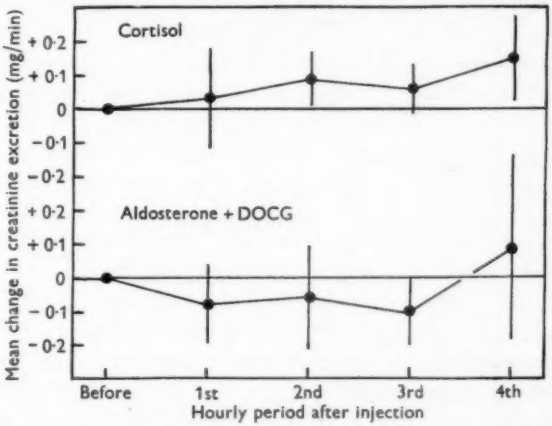

Fig. 5. Afternoon expts., subjects A, F, K, M and T. Mean change in creatinine output in each successive hour after steroid injection, with reference to the hour before injection. Above, after cortisol; below, after aldosterone and DOCG. Vertical lines indicate 95% confidence limits of the mean change.

bicarbonate; and since in addition it is uncertain whether all bicarbonate reabsorption is dependent upon hydrion secretion, it is impossible to make any calculations of hydrion secretion in these experiments, although it was certainly low. Neither steroid had any consistent effect upon bicarbonate excretion.

The high urinary bicarbonate was associated with a copious excretion of sodium and potassium. All subjects at some period were excreting sodium at a rate in excess of 380, and potassium in excess of 140, μequiv/min, and the highest rates were 801 μequiv/min of Na and 373 μequiv/min of K. It is upon the relative contributions made by these two cations that the steroids appear to have exerted their effect. The Na:K ratio (Fig. 6) remained high in all control experiments, with usually a tendency to a progressive rise. After injection of aldosterone or cortisol this ratio fell

abruptly and was still low at the end of the observations. No discrimination between the action of the two hormones was thus possible. Neither steroid caused any consistent change in creatinine excretion.

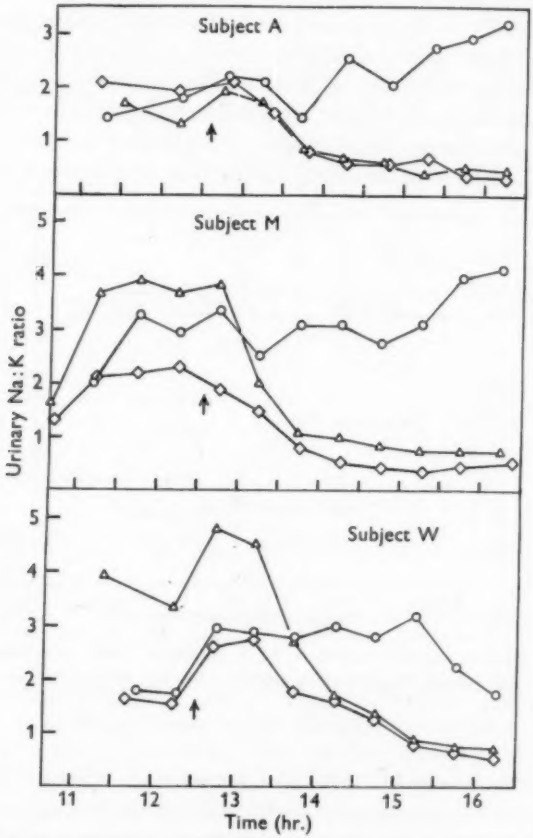

Fig. 6. Acetazoleamide expts., subjects A, M and W. Urinary Na:K ratio: ○, control; ◇, aldosterone; △, cortisol. Arrow indicates time of injection of acetazoleamide, with or without steroid, except that in subject M acetazoleamide was taken by mouth at 11.00 hr, and arrow indicates time of injection of steroids only.

Phosphate excretion was raised by acetazoleamide to values 15–20 μmole/min above those observed in control experiments. Cortisol depressed this output sharply, but not to such low values as were found when cortisol was given without acetazoleamide.

Time course of steroid action

An attempt has been made to assess, by visual inspection of plots of urinary variables against time, the delay between intravenous injection of steroids and onset of change in renal cation handling. In afternoon and acetazoleamide experiments, the ratio Na:K was the most sensitive index of hormone activity, but in the morning this ratio was rather variable, and Na:*B* provided a better criterion. The delay was assessed in all experiments where controls were available for comparison: sixteen afternoon, seven morning and six acetazoleamide. The values ranged from $\frac{1}{2}$ to $1\frac{3}{4}$ hr with a mean of 54 min, probable limits ($P = 0{\cdot}05$) 45–63 min. The scatter of values is probably due, in part, to the difficulty of accurate assessment of the delay. No evidence was found that the delay was affected by the steroid used, or by the dose of aldosterone (0·25 or 0·125 mg of the D-isomer).

Little can be said about the duration of action of the steroids, since there was seldom any sign of diminution of action when observations ended, 4 hr after injection. In one experiment, after a single dose of 0·25 mg D-aldosterone, activity was clearly diminishing after 3 or 4 hr.

There were certain indications that the action of cortisol might change qualitatively after some hours, so that different criteria might suggest a different duration of action. Thus, despite a continued sodium retention, the potassium excretion after cortisol injection did not always remain high, (Subject T, Figs. 1, 2 and 3), and when it fell, hydrion output rose. The time course of the diminished phosphate excretion after cortisol injection is much briefer than that of the changes in cation excretion. Mills & Thomas (1958) made the same comment about the changes in plasma phosphate concentration, and these changes in phosphate metabolism are apparently extrarenal (Mills & Thomas, 1959).

Night experiments

During the night the excretion of potassium is usually very low, and that of hydrion high, with sodium output more variable but usually also low (Black & Mills, 1954). Fairly consistent levels of excretion are observed, at least for some subjects, on a series of nights (compare Figs. 3, 2 and 1 of Black & Mills, 1954, with Fig. 7 of this paper). The action of steroids has therefore been studied against this background, which is very different from that during the afternoon. Five subjects were used. Figure 7 shows the outputs of Na, K and hydrion (H′), and the mean effects of the steroids upon each subject are shown in Table 3. In order to assess, on the five subjects collectively, the significance of the effects ascribed to steroid administration, analysis of variance (Rao, 1952) has been applied to the

data for Na, K, H′ and creatinine outputs; the P values in the next paragraphs are those derived from this analysis.

Both aldosterone and cortisol produced a decreased excretion of sodium of about 20 μequiv/min ($0{\cdot}01 > P > 0{\cdot}001$ and $0{\cdot}05 > P > 0{\cdot}025$, respectively); the difference between the effects of aldosterone and cortisol was

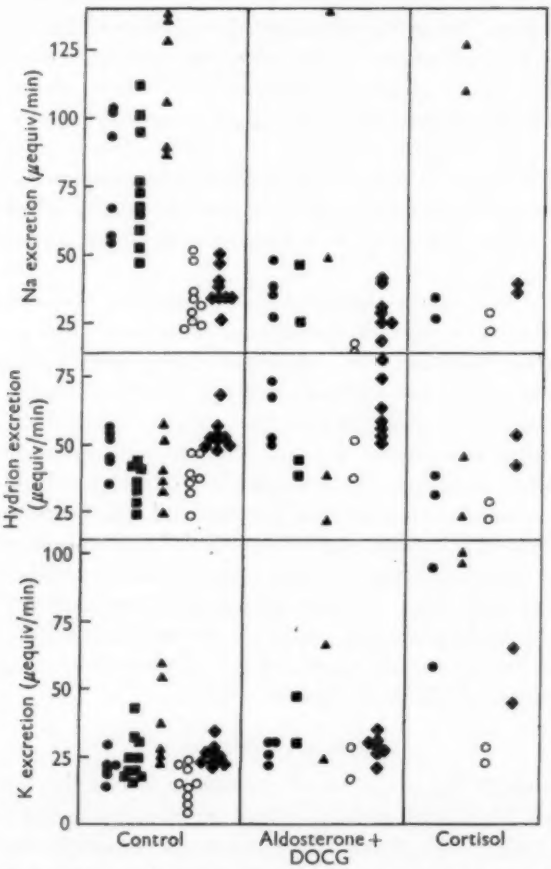

Fig. 7. Night expts., urinary sodium, potassium and hydrion (H′) outputs: ●, subject H; ■, subject F; ▲, subject I; ○, subject T; ◆, subject W.

not significant ($P > 0{\cdot}05$). Potassium output was only slightly increased by aldosterone (mean 6 μequiv/min, $0{\cdot}025 > P > 0{\cdot}01$), in contrast with a large increase following cortisol (mean 32 μequiv/min, $P < 0{\cdot}001$). The difference in effect of the two steroids was significant ($P < 0{\cdot}001$). The fall in Na and rise in K output after all steroids depressed the Na:K ratio

(Fig. 8). The depression tends to be somewhat greater after cortisol, presumably due to the greater elevation of K output.

The mean change in hydrion output (H′) was a rise of 6 μequiv/min after aldosterone ($0{\cdot}05 > P > 0{\cdot}025$), and a fall of 10 μequiv/min after cortisol ($0{\cdot}01 > P > 0{\cdot}001$). Titratable acidity was measured in all steroid experiments, though not in all controls; in calculating the difference between the steroids the more adequate expression of hydrion output (H) can be used. Thus calculated, hydrion output after aldosterone exceeded that after cortisol injection by a mean of 19 ± 5 μequiv/min.

TABLE 3. Mean increase in urinary night outputs after different steroids. Where the variable is a ratio (Na:K, h′:K) the figure given is the ratio of the geometric means of the two series of experiments

	Subject	Na	K	H*	Creatinine	Na:K	h′:K	Phosphate
ldosterone and DOCG–control	F	−39	+14	+ 6	+0·21	0·30	1·09	+1
	H	−42	+ 6	+14	+0·05	0·35	0·92	+2
	I	−20	+ 8	−14	+0·04	0·66	0·67	−2
	T	−18	+ 8	+ 6	−0·01	0·32	0·70	+2
	W	− 8	+ 3	+10	+0·05	0·69	1·03	+2
	W¹	+ 3	+11	+17	—	0·71	—	—
	W²	−13	+ 3	+18	—	0·55	—	—
Weighted mean		-19 ± 4	$+ 6 \pm 2$	$+ 6 \pm 3$	—	—	0·95	—
ortisol–Control	H	−49	+56	−15	+0·03	0·10	0·17	−6
	I	0	+60	+ 1	+0·08	0·29	0·34	−8
	T	−10	+12	−13	+0·02	0·39	0·32	−7
	W	0	+29	− 6	−0·04	0·48	0·43	−15
Weighted mean	—	-18 ± 9	$+32 \pm 3$	-10 ± 3	—	—	0·353	—
ldosterone and DOCG–cortisol	H	—	−50	+29	—	—	4·22	—
	I	—	−53	−15	—	—	2·00	—
	T	—	− 4	+24	—	—	2·19	—
	W	—	−26	+16	—	—	2·40	—
Weighted mean	—	—	-26 ± 4	$+19 \pm 5$	—	—	—	—

W¹ and W² refer to the first and the last half of a night in which the urine collection was divided.

* Owing to the lack of data on titratable acidity of the control nights on subjects F and T, titratable cid phosphate has been used in all calculations involving controls on these subjects.

In Table 3 it may be observed that after aldosterone or cortisol the changes in Na, K and hydrion add together to nearly zero. This suggests that the effect of both steroids was upon cation exchange in the renal tubule; the total cation exchanging was, however, considerably greater under the influence of cortisol. The pattern of the exchange produced by the different steroids was dissimilar; cortisol produced a much greater increase in K output and depressed, instead of elevating, hydrion output. This difference is clearly demonstrated, as in the afternoon experiments, by the hydrion:K ratios shown in Table 3 and Fig. 8. As with the Na:K ratio, the statistical significance of the differences and the weighted mean effect have been calculated from the logarithms. h′:K ratio was in no subject significantly altered by aldosterone but it was grossly reduced by cortisol, exactly as in the afternoon experiments.

The effect of a single intravenous injection of aldosterone may decrease within 4 hr (see above). In all except two experiments aldosterone was injected intramuscularly at bedtime, to prolong its action, but the two intravenous injections had much the same effect. As a check upon the time course, further experiments, three controls and three with aldosterone

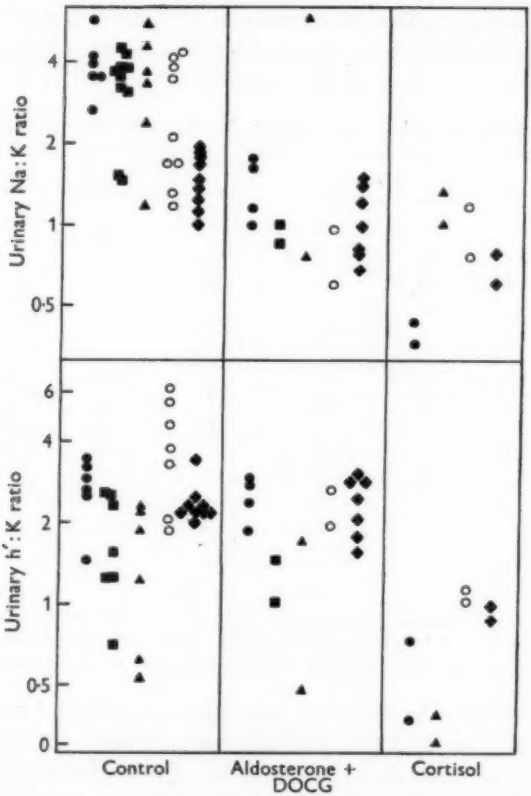

Fig. 8. Night expts., urinary Na:K and h′:K ratios, log. scale. Symbols as in Fig. 7.

injection, were performed upon subject W, in whom the effect of aldosterone appeared minimal. He was awakened by an alarm clock after 4 hr of sleep and passed urine, thus dividing the night's collection into 2 portions. The mean differences between aldosterone and controls are included in Table 3. The only significant effect was a rise in K output in the earlier half of the night, but this was much smaller than that produced by cortisol over the whole night. There is little suggestion that useful information is lost by

pooling the urine of a whole night. The outputs of the whole night covered by these experiments have, therefore, been calculated and included with the figures for W during a continuous night's sleep.

As by day, phosphate excretion was unaffected by aldosterone (Table 3). Cortisol depressed phosphate excretion; the fall was less notable than by day, but this is probably due to the pooling of 8 hr urine, since the time course of the lowered phosphate excretion after cortisol is brief (Fig. 4).

Creatinine outputs were measured in nearly all night experiments, as an indication of the G.F.R. The means of control nights were 1·09, 1·34, 1·28, 1·01 and 1·04 mg/min in subjects F, H, I, T and W, respectively. The changes due to steroids are shown in Table 3; all were trifling and analysis of variance shows them to be not significant.

Morning experiments

A series of control cortisol and aldosterone experiments in the morning was performed upon subjects C, P and W. The pattern of electrolyte excretion immediately before steroid injection, at 9.30 hr, was exceedingly variable; sodium outputs were as low as 46 and as high as 253 μequiv/min on different occasions on a single subject, and potassium outputs covered nearly as wide a range. There was a suggestion of a difference between the action of cortisol and aldosterone, similar to that observed in the afternoon, but the results are difficult to assess, owing to the wide variability of the starting point. For this reason, it appeared that no firm conclusions could be drawn without considerable multiplication of morning experiments.

DISCUSSION

Vander, Malvin, Wilde, Lapides, Sullivan & McMurray (1958) have shown by the stopped flow technique that, at least in the dog, one site of action of aldosterone is the distal renal tubule. We suggest that cortisol differs in its action upon cation exchange at this site. Three other possible mediators of altered cation excretion must, however, first be excluded: changes in glomerular filtration or in buffer excretion, and extrarenal actions. Different steroids might mediate their effects in any of these three ways.

Adrenal steroids can alter Na output by changing G.F.R. (Green, Johnson, Bridges, Lehmann & Gray, 1950). Creatinine output, which we have used as a rough indication of G.F.R., rose slightly after cortisol in the afternoon and perhaps fell after aldosterone, but the effects on sodium excretion were indistinguishable. By night creatinine excretion was not affected by steroids though sodium excretion fell. Mills & Thomas (1958) observed consistent effects of cortisol upon Na retention despite very variable effects

upon G.F.R. and renal plasma flow. Na output after adrenal steroids does not thus appear to be mainly determined by changes in G.F.R.

Cortisol depresses phosphate excretion by lowering plasma concentration, probably mainly through diversion of phosphate into muscle (Mills & Thomas, 1959). Since phosphate is a major urinary buffer, accounting for about half the titratable acidity of acid urines (Wrong & Davies, 1959; our own unpublished observations), decreased phosphate excretion may well lead to reduced excretion of hydrion. Schiess, Ayer, Lotspeich & Pitts (1948) have shown that in man excretion of titratable acid increases linearly with output of any particular buffer; they did not, however, measure any other component of hydrion output, and there is no evidence that total urinary hydrion excretion is dependent on the amount of urinary buffer, particularly when this is normal or diminished. It may well be that a fall in the amount of urinary buffer will merely result in the diversion of more secreted hydrion into other channels, namely, combination with ammonia or with bicarbonate ions.

If cortisol stimulates hydrion secretion to the same extent as do aldosterone and DOCG, but the response is limited by deficiency of buffer, then one would expect the pH of the urine to remain unaltered or more probably to fall. Since, however, in afternoon experiments cortisol elevated the urinary pH, the reduced hydrion excretion after its injection is not determined by a low buffer excretion. In night experiments the drop in phosphate excretion after cortisol injection was small and could hardly account for a difference of 19 μequiv/min in hydrion output, nor could lack of available buffer easily explain the greater over-all increase in cation exchange observed after cortisol injection.

Bartter & Fourman (1957), whose observations resembled our own, ascribed the kaliuresis after cortisol to a rise in plasma potassium concentration, due perhaps to increased tissue break-down; Knight, Kornfeld, Glaser & Bondy (1955) also found a rise of plasma potassium concentration after giving cortisol, but other workers have found the concentration to remain unchanged or to fall (Frawley, 1955; Raisz, McNeely, Saxon & Rosenbaum, 1957; Mills & Thomas, 1957). Figure 9 of Mills & Thomas (1957) includes plasma potassium concentrations for subjects M and F in the cortisol experiments here recorded, so that rising excretion and falling plasma concentration have been observed simultaneously. There is thus no support for an extra-renal origin of the kaliuresis. One might expect that an extrarenal action would still be observed in subjects given acetazoleamide.

Reports in the literature of acute steroid effects in man, fully controlled, are few. The somewhat erratic results of Huffman, Wilson, Clark & Smyth (1956) and of Knight *et al.* (1955), with intravenous doses of cortisol

similar to ours, may be due to insufficient allowance for the large spontaneous variability of Na output, particularly in the morning. There is, however, sufficient evidence in the literature for acute sodium retention under the influence of all steroids here considered.

Many workers have observed a rise in potassium excretion after giving cortisol to human subjects (Fourman, Bartter, Albright, Dempsey, Carroll & Alexander, 1950; Knight *et al.* 1955; Bartter & Fourman, 1957; Jefferies, Kelly, Sydnor, Levy & Cooper, 1957; Dingman, Finkenstaedt, Laidlaw, Renold, Jenkins, Merrill & Thorn, 1958). Raisz *et al.* (1957) described the change of potassium output as modest, but they made their observations over a time of day when potassium excretion was falling steeply in control experiments; inspection of their data shows that after cortisol injection potassium excretion rose to a level two or three times as great as in control experiments, and that the increase in potassium output was at least as great as the sodium retention. By contrast, an acute rise of potassium excretion has been less regularly observed after DOC or aldosterone, and has usually been smaller than the fall in sodium excretion (Fourman, Reifenstein, Kepler, Dempsey, Bartter & Albright, 1952; Sheppard, Morse, Renold & Thorn, 1955; Thorn, Sheppard, Morse, Reddy, Beigelman & Renold, 1955; Liddle, 1958).

Berliner (1950) first suggested that adrenal steroids might stimulate the ion exchange mechanism whereby the distal tubule is supposed to secrete potassium in exchange for reabsorbed sodium (Berliner, Kennedy & Hilton, 1950). This suggestion has been widely accepted, though usually only by implication. The possibility that adrenal steroids stimulate sodium reabsorption in exchange for secreted hydrion, the mechanism proposed by Pitts & Alexander (1945), has been considered by Pitts (1952), Knight *et al.* (1955), Bartter (1956), Dingman *et al.* (1958), Perlmutt & Olewine (1958), and Liddle (1958). Only Bartter & Fourman (1957), however, mention the possibility that different steroids may differ in their action upon these two cation exchange mechanisms; and we have found no report in which all the separate indications of hydrion exchange—urinary pH, ammonium output, titratable acid and bicarbonate—have been measured in human subjects after intravenous administration of steroids. No detailed comparison with the findings of other workers is thus possible.

Numerous observations over more prolonged periods, such as days or weeks, suggest differences between the action of different steroids on electrolyte balance. These are, however, outside the scope of the present discussion, since over long periods many secondary effects may obscure the direct renal actions.

Findings similar to our own have been reported on other species. Desaulles (1958), working with adrenalectomized rats, found that for a

similar sodium retention corticosterone and cortisol caused much greater excretion of potassium than did aldosterone or DOC. Perlmutt & Olewine (1958), also using rats, found that DOCG depressed Na output without increasing excretion of potassium; after acetazoleamide and DOCG, however, the fall in Na excretion was paralleled by a rise in potassium excretion.

A quite distinct method of investigating the renal effects of aldosterone is to observe the immediate effects of a blocking agent such as SC–8109 (Searle), which is without direct effect on renal function but prevents the action of aldosterone (Kagawa, Cella & Van Arman, 1957; Liddle, 1957; Ross & Bethune, 1959). In circumstances where aldosterone secretion is presumed to be considerable, this drug has been found to diminish excretion of titratable acid and ammonium (Liddle, 1958; McCrory & Eberlein, 1958; Wiggins, Hutchin, Carbone & Doolan, 1959), as well as increasing sodium and decreasing potassium excretion. Confirmation is thus provided for our observation that aldosterone can stimulate acid as well as K excretion by the kidney.

The findings can all be explained by supposing that the direct action of cortisol is to stimulate potassium secretion in exchange for sodium. This usually leads to a substantial fall in sodium excretion, but if sodium reabsorption is already nearly complete, as in some subjects at night, then the potassium secretion is at the expense of hydrion secretion (Berliner, Kennedy & Orloff, 1951). Aldosterone and DOC, on this hypothesis, stimulate sodium reabsorption, in exchange for whatever cation is available; both in the afternoon and at night, excretion of hydrion and of K increase in the same proportion. When, however, hydrion secretion is blocked by acetazoleamide, reabsorption is largely or entirely in exchange for potassium. Under other circumstances it is still not known what determines the partition of secretion between potassium and hydrion, but it varies spontaneously over the twenty-four hours (Mills & Stanbury, 1954). A part of this 24 hr cycle is shown in the afternoon control experiments; the behaviour of subject T (Fig. 3) is typical. A further portion is represented by the night experiments. It will be seen that, although aldosterone and deoxycorticosterone increase the total potassium and hydrion secretion, the proportional distribution between the two continues to follow the habitual diurnal rhythm.

SUMMARY

1. Urine was collected from healthy men after injection of cortisol, aldosterone, or deoxycorticosterone, and compared with collections from the same subjects without injection. The doses of steroid used produced similar Na retention.

2. In the afternoon cortisol increased K output by an amount similar to the Na retention, and hardly altered hydrion output. Hydrion:K excretory ratio was thus grossly reduced. Aldosterone and deoxycorticosterone increased excretion of both hydrion and K, and the hydrion:K ratio followed a similar course to that observed in control experiments.

3. When steroids were given at bedtime and the night's urine collected, cortisol caused a rise of K excretion much greater than the Na retention, balanced by a fall in hydrion excretion. Aldosterone and deoxycorticosterone, as in the afternoon, increased outputs of hydrion and K but left the hydrion:K ratio unaltered.

4. In subjects with hydrion secretion blocked by acetazoleamide, the difference between the steroids disappeared: all steroids reduced Na and increased K excretion.

5. In morning experiments random variations in outputs were too great to permit discrimination between the effects of the steroids.

6. Changes in creatinine excretion were trifling after all steroids.

7. It is suggested that cortisol stimulates K excretion, resulting in either Na retention or diminished hydrion $\rightleftharpoons$ Na exchange, whilst aldosterone stimulates Na reabsorption in exchange for K and hydrion in whatever proportions are available.

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THE EFFECT OF SYMPATHETIC STIMULATION ON MAMMALIAN MUSCLE SPINDLES

By C. C. HUNT

From the Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.

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Several authors have described unmyelinated fibres entering the mammalian spindle (see Barker, 1948). These fibres are reported to degenerate on removal of the appropriate sympathetic ganglia (Hines & Tower, 1928) and are the only fibres to remain intact after complete degeneration of the somatic innervation to the spindle (Hinsey, 1927). Although the characteristics of the discharge from muscle spindle afferent fibres and the influence thereon of motor fibres to the mammalian spindle (fusimotor fibres) have been studied in detail (Matthews, 1933; Leksell, 1945; Kuffler, Hunt & Quilliam, 1951; Hunt & Kuffler, 1951), the physiological role of sympathetic fibres to the spindle has not yet been described. A preliminary report of the present results was presented at the September 1959 meeting of the American Physiological Society (*Physiologist*, **2**, 61–62).

In the present experiments the effects of stimulating sympathetic fibres on the discharge frequency in spindle afferent fibres, on the threshold to stretch of spindle receptors, and on the afferent spindle response to fusimotor stimulation, have been studied in the cat. It will be shown that sympathetic stimulation results in significant changes in threshold of spindle afferent endings in the form of an initial facilitation and subsequent depression. These threshold changes are manifest in changes in discharge frequency and in responses to fusimotor stimulation under conditions of constant muscle tension.

METHODS

Adult cats anaesthetized with sodium pentobarbital were used. With the animal lying on its side the abdominal sympathetic trunk was exposed retroperitoneally and cut in the mid-lumbar region. The distal portion was freed for subsequent placement on stimulating electrodes. Laminectomy was performed to expose the lumbosacral cord and spinal roots. Dorsal and ventral roots from L 6 to S 2 were cut close to the cord. Nerves in the limb were cut, save that to the muscle under study. The muscle tendon was cut and attached to a strain gauge or puller. Discharge in single afferent fibres from the muscle was recorded in dorsal root filaments and individual fusimotor fibres to the muscle were stimulated in ventral root filaments. Stimulation of the sympathetic trunk was carried out with rectangular

pulses of 1 msec duration. Pilomotor responses served as a useful index of the adequacy of sympathetic stimulation. Spindles from gastrocnemius, soleus and tenuissimus muscles were studied.

The device for producing muscle stretch (see Appendix) permitted a stretch to be applied which increased linearly with time and which could be controlled as to magnitude and duration. A vane attached in series between the tendon and puller was interposed between a diffused light source and a photocell. The output of the photocell was recorded on one beam of a Tektronix 502 oscilloscope. Threshold to stretch of a spindle receptor could be estimated by the amount of muscle displacement required to initiate an impulse. This was determined by simultaneous photography of a stretch-evoked impulse in an afferent fibre and the extent of muscle stretch as measured by the photocell.

Tissues were covered with pools of paraffin oil initially equilibrated with 95% O and 5% CO_2. Temperature was maintained at 37–39° C.

RESULTS

Afferent discharge at constant muscle tension

In a spindle afferent fibre showing base-line discharge at constant muscle tension, sympathetic stimulation caused an initial increase followed by a decrease or cessation of discharge. This effect occurred after a considerable latent period and depended on the duration and frequency of sympathetic

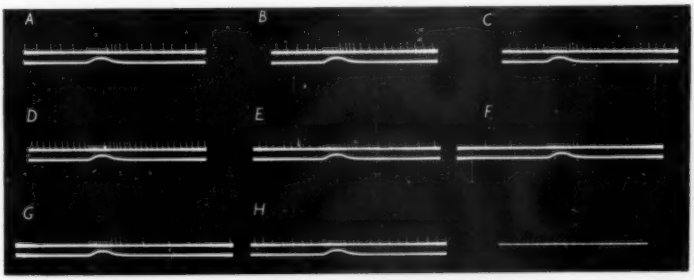

Fig. 1. Portions of continuous record from dorsal root filament (upper trace) containing one muscle spindle afferent fibre and one tendon organ fibre; lower trace, muscle tension recorded by strain gauge. *A* control. Between *A* and *B* sympathetic stimulation at 41/sec begun (total duration 16·2 sec). *B* 14·5 sec after onset of sympathetic stimulation. *C–H* at the following times after end of sympathetic stimulation: *C* 0 sec, *D* 6·7 sec, *E* 8·5 sec, *F* 10 sec, *G* 11·8 sec and *H* 17 sec. Time trace lower right, 10 msec intervals. Soleus muscle under slight initial tension.

stimulation. A typical experiment is shown in Fig. 1. The discharges in a muscle spindle afferent fibre and in a tendon organ fibre (from soleus) were recorded in the same dorsal root filament on continuously moving film. Contraction of the muscle was produced by single stimuli to the ventral roots supplying the muscle. The spindle discharge showed typical cessation during the muscle twitch, whereas the tendon organ fibre showed discharge only during contraction. Record *A* represents a segment of the record in the control period. Sympathetic stimulation at 41/sec was then

applied for 16·2 sec. Record *B* was from a period near the end of sympathetic stimulation, and record *C* just after stimulation had ceased. *D*, taken 6·7 sec after cessation of sympathetic stimulation, shows an obvious increase in the frequency of the spindle afferent discharge. Later the record showed slowing or cessation of the spindle discharge (*F* and *G*), followed by return toward the control frequency (*H*). Throughout this period there was no change in the tendon organ discharge. A number of tendon organ receptors have been examined, none of which were influenced by sympathetic stimulation.

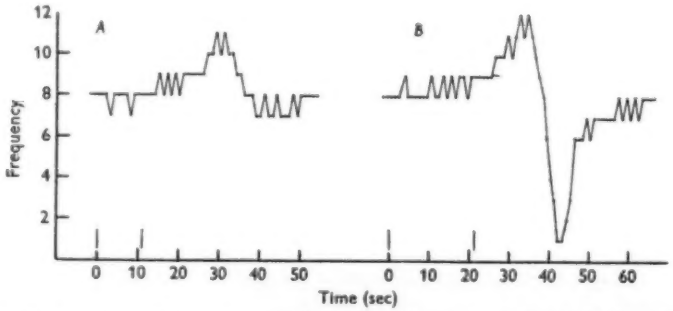

Fig. 2. Frequency changes in an afferent fibre from a muscle spindle in soleus. Sympathetic stimulation (100/sec) for 11 sec in *A* and 21 sec in *B*. Period of stimulation noted by vertical lines above abscissa.

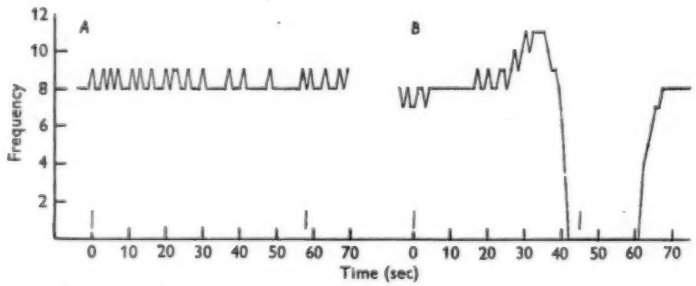

Fig. 3. Frequency changes in same fibre as in Fig. 2. In *A* sympathetic stimulation at 1·75/sec for duration indicated between vertical lines. In *B* sympathetic stimulation at 2/sec for period indicated.

The effect on the afferent discharge from muscle spindles could be graded by the frequency and duration of sympathetic stimulation. Figure 2 shows frequency plots of the response of a spindle afferent fibre (soleus) to sympathetic stimulation at 100/sec. In *A* stimulation for 11 sec was followed by an increase in discharge frequency. In *B* stimulation for 22 sec caused an initial increase followed by a decrease in frequency. More prolonged

sympathetic stimulation resulted in an initial increase, then a period of cessation of afferent discharge, followed by recovery (Fig. 3*B*). The optimal frequency of sympathetic stimulation for the production of the above changes was usually between 10 and 30/sec.

The lowest effective frequency of sympathetic stimulation was often quite critical. For example, the spindle afferent fibre shown in Fig. 3 showed no effect from sympathetic stimulation at 1·75/sec for 58 sec (*A*), but stimulation at 2/sec (*B*) for 45 sec caused the typical sequence of initial increase followed by cessation of afferent discharge and subsequent recovery. The lowest effective frequency of sympathetic stimulation was usually between 2/sec and 5/sec. These results suggest that the accumulation of a substance released by sympathetic stimulation is necessary to cause the alterations in sensory discharge (see Discussion).

The changes in afferent discharge from muscle spindles, as described above, were also found in the tenuissimus muscle suspended in the oil pool and devoid of circulation. This was confirmed in several experiments, in which microscopic observation showed the lack of blood flow in vessels within the muscle. This finding indicates that the changes in spindle discharge are not secondary to alterations in blood flow.

Threshold of spindle afferent endings. The threshold of spindle receptors to stretch following sympathetic stimulation was estimated by linearly increasing muscle stretch as described in Methods. Figure 4 illustrates a typical result in a fibre from triceps surae which showed no base-line discharge at the initial tension employed. Control records are shown in *A* and *B*, the impulse occurring consistently at a certain degree of stretch. Between *B* and *C* sympathetic stimulation was begun (tetanus at 10/sec lasting 28 sec). The threshold was reduced in *C*, *D* and *E*, the latter record showing two impulses as a result of the standard stretch. Records *F* and *G* show the subsequent increase in stretch threshold and in record *H* the amount of stretch applied failed to initiate an impulse. Records *I*–*M* show the recovery to the control stretch threshold.

The threshold to stretch was measured by noting the extent of muscle displacement as recorded in the photocell record at the time of occurrence of the impulse (conduction time was small relative to duration of stretch). The threshold was then plotted as a function of time. Figure 5 shows the threshold changes resulting from sympathetic stimulation as recorded in a single spindle afferent fibre. In *A* the sympathetic trunk was stimulated at 30/sec for 44 sec at a given stimulus strength. The stretch threshold showed an initial fall followed by a sharp rise. The receptor then failed to initiate an impulse with the degree of stretch applied by the puller. However, if a greater degree of stretch were employed, the receptor could be made to discharge during this period. After cessation of sympathetic

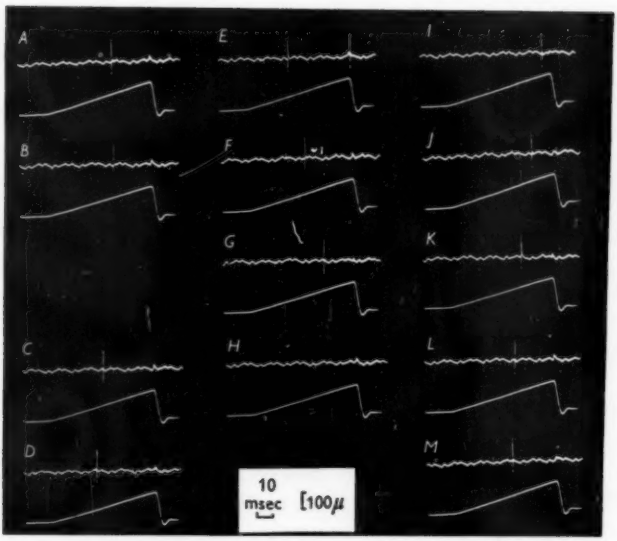

Fig. 4. Responses to stretch in a spindle afferent fibre from triceps surae. Upper record, discharge in afferent fibre; lower record, displacement of muscle. Low initial tension, no base-line discharge from spindle. *A* and *B*, controls. Sympathetic stimulation at 10/sec begun between *B* and *C*, ended between *H* and *I* (total duration 28 sec). Records *C*–*I* at the following times after onset of sympathetic stimulation: *C* 10 sec, *D* 14 sec, *E* 16 sec, *F* 20 sec, *G* 22 sec, *H* 26 sec. Records *I*–*M* at the following times after cessation of sympathetic stimulation: *I* 16 sec, *J* 18 sec, *K* 20 sec, *L* 22 sec, *M* 26 sec. Sweep duration 100 msec. Ordinate applies to lower traces.

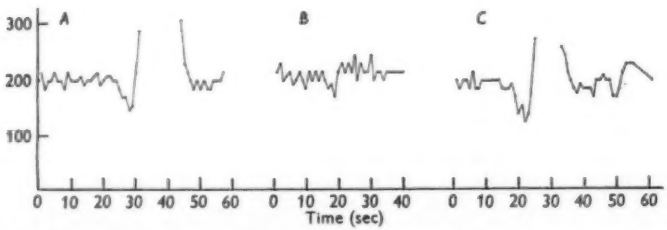

Fig. 5. Threshold changes of a spindle receptor to stretch following sympathetic stimulation. Ordinate shows extent of muscle stretch (μ) associated with impulse initiation. *A*, sympathetic stimulation 30/sec for 44 sec at strength 7 V. *B*, sympathetic stimulation 30/sec for 10 sec at strength 7 V. *C*, sympathetic stimulation 30/sec for 10 sec at strength 14 V; spindle from triceps surae (fibre conducted at approximately 52 m/sec); data derived from experiment similar to that of Fig. 4. Stimulation begins at time zero in *A*, *B* and *C*.

stimulation the threshold returned to normal. These threshold changes mirror the changes in discharge frequency described above. Figure 5*B* illustrates the effect produced by stimulation at the same strength and frequency for a shorter duration, namely, 10 sec. Only slight initial fall in stretch threshold occurred. In Fig. 5*C* stimulation was for the same duration and at the same frequency as in *B* but the stimulus strength was doubled. This resulted in striking threshold changes, comparable to those in *A*. The increase in the effect of sympathetic stimulation consequent to the change in stimulus strength between *B* and *C* indicates that convergence of more than one sympathetic efferent fibre may occur to the same spindle.

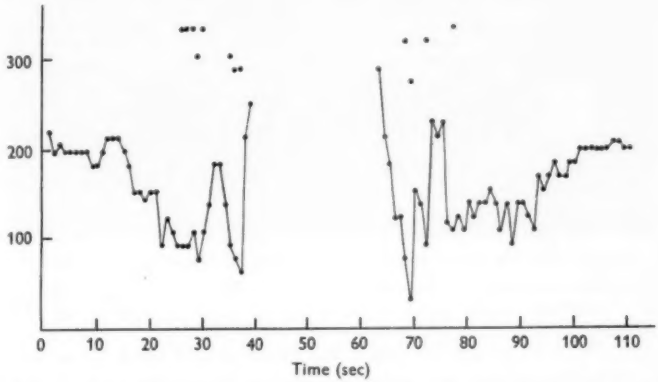

Fig. 6. Threshold changes (μ) of same unit as in Fig. 5 to intravenous injection of 200 μg adrenaline, given at time 10 sec. Dots indicate extent of muscle stretch associated with second impulse when this occurred. Stretch employed as in Fig. 4.

The effects of sympathetic stimulation on the stretch threshold of spindle receptors were similar to those occurring after intravenous injection of adrenaline although the time course was slightly different. Figure 6 shows the changes in stretch threshold of a spindle receptor that occurred following the intravenous injection of 200 μg of adrenaline. Typically a fall in threshold was followed by a marked rise in threshold. On recovery there was often a phase of enhanced excitability before the threshold returned to normal. A similar but less conspicuous second phase of decreased threshold before recovery was sometimes seen following sympathetic stimulation. The doses of adrenaline injected intravenously that were required to produce significant threshold changes were usually about 40–80 μg/kg. Intravenous adrenaline also produced changes in discharge frequency in spindle afferent fibres similar to those which followed sympathetic stimulation.

Responses to fusimotor stimulation. As might be expected from the changes produced by sympathetic stimulation on stretch threshold of spindle receptors, alterations in the spindle response to fusimotor stimulation also occurred in response to sympathetic activity. Figure 7 shows the response to fusimotor stimulation in a spindle afferent fibre from medial gastrocnemius that has no base-line discharge. In each record 11 stimuli were delivered to a fusimotor fibre isolated to this spindle at 100/sec; no detectable contraction of the muscle could be observed. *A* and *B* represent control responses. Sympathetic stimulation (30/sec) was begun between *B* and *C* and was discontinued between *E* and *F*, the total

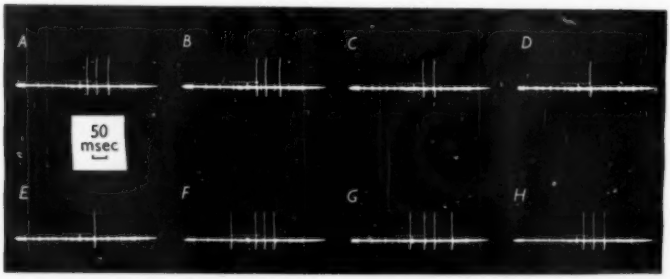

Fig. 7. Effect of sympathetic stimulation on responses in a spindle afferent fibre from medial gastrocnemius to stimulation of a fusimotor fibre (11 stimuli at 100/sec) isolated in ventral root. At the constant low initial tension employed, no base-line discharge was present in the afferent fibre. *A* and *B*: control responses. Between *B* and *C* sympathetic stimulation at 30/sec was begun (total duration 90 sec). Records *C–E* at the following times after the onset of sympathetic stimulation: *C* 60 sec, *D* 64 sec and *E* 88 sec. Records *F–H* at the following times after cessation of sympathetic stimulation: *F* 30 sec, *G* 50 sec and *H* 90 sec.

duration of stimulation being 90 sec. It may be seen that sympathetic stimulation caused a decrease in the response to fusimotor stimulation (*C* to *E*) with some possible facilitation during recovery (*F* and *G*). In *H*, taken 90 sec after cessation of sympathetic stimulation, the response has returned to normal. These changes in response to fusimotor stimulation probably reflect the changes in receptor threshold that follow sympathetic stimulation. However, an initial phase of facilitation was not as conspicuous in fusimotor responses as in threshold changes.

General comment

The great majority of spindles examined showed the effects noted. In later experiments typical effects of sympathetic stimulation were almost invariable. Some early failures were attributed to excessive cleaning of the muscle nerve. In a number of the spindle afferent fibres studied conduction velocity was determined. Typical effects of sympathetic

stimulation, as described above, were seen in spindle afferent fibres conducting at velocities characteristic of both Group I (72–120 m/sec) and Group II (30–72 m/sec) fibres (Hunt, 1954).

DISCUSSION

Sympathetic fibres are known to enter the muscle spindle and to terminate within the capsule in the region of the sensory terminals (Barker, 1948). Activity in such sympathetic fibres has been shown in the present study to cause significant changes in the threshold of spindle receptors to applied stretch. The effect of sympathetic stimulation on spindle receptor threshold is a dual one, the initial decrease in threshold being followed by an increase. The changes in discharge frequency in a spindle afferent fibre showing base-line discharge to a fixed degree of external stretch may be attributed to the threshold variations that occur in the receptor. Likewise the alterations in response to fusimotor activity following sympathetic stimulation appear to have a similar cause. An alternative explanation would be that activity in sympathetic fibres to the spindle causes a change in the intrafusal muscle fibres. This would have to occur in the absence of efferent discharge in the motor fibres to the spindle and would be analogous to the effects of the sympathetic on tonus of certain smooth muscles. However, there is no evidence to suggest that intrafusal fibres differ from extrafusal fibres by possessing such tonic properties. Furthermore, there is suggestive evidence that adrenaline may affect nerve endings. Adrenaline causes an increase in frequency of miniature end-plate potentials (Krnjević & Miledi, 1958); this could be a consequence of depolarization of the motor nerve endings. Also Lowenstein (1956) noted a lowering of threshold of receptors in frog skin on stimulating the sympathetic trunk. He also found that the effects of sympathetic stimulation could be reproduced by administration of adrenaline or noradrenaline.

One may postulate that the sympathetic endings in the spindle release a substance on activity and that this substance, possibly adrenaline and/or noradrenaline, on reaching an appropriate concentration, causes a decrease in receptor threshold, and at higher concentrations causes an increase in threshold. Repetitive stimulation of the sympathetic would then be necessary to permit the accumulation of a sufficient concentration of the substance released. Such a scheme could explain the critical minimal effective frequency of sympathetic stimulation, and the dependence of the effects on frequency and duration of sympathetic stimulation

While prolonged sympathetic activity is required to affect spindle receptor threshold, the effective frequences are low. Hence tonic discharge in sympathetic efferent fibres could produce significant effects on spindle

receptors. The functional role of this accessory innervation to the muscle spindles is not clear. While some role could be attributed to the decrease in receptor threshold as a consequence of general sympathetic activity, it is difficult to deduce a meaningful function for the increase in threshold which usually follows.

SUMMARY

1. The effect of stimulation of the sympathetic trunk on muscle spindles has been studied in the cat.

2. Repetitive sympathetic stimulation of relatively long duration but of low or moderate frequency results in an initial lowering of threshold to stretch followed by an increase of threshold that may last for many seconds. The magnitude of the changes depends upon the frequency and duration of the tetanus applied to the sympathetic fibres.

3. With the muscle under constant tension, sympathetic stimulation produces an initial rise followed by a fall in discharge frequency in spindle afferent fibres. This effect parallels the changes in stretch threshold of spindle receptors.

4. Sympathetic stimulation alters the response of spindle afferent fibres to fusimotor stimulation, the most conspicuous change being a reduction in the number of responses to a standard tetanus to a fusimotor fibre. This effect is also attributed to receptor threshold changes.

5. The above effects of sympathetic stimulation can be imitated by injection of adrenaline intravenously.

6. No changes in tendon organ discharge have been found following sympathetic stimulation.

7. The effects of sympathetic stimulation on muscle spindles also occur in the tenuissimus muscle deprived of its circulation, and hence are not attributable to alterations in blood flow.

The valuable technical assistance of Miss Ann Stephen is gratefully acknowledged. This work was supported by a research grant (B-1320) from the U.S. Public Health Service, National Institutes of Health.

APPENDIX

A control circuit for an electromagnetic puller

By H. Fein

The electromagnetic puller (Goodmans Industries Ltd. Model V47) is excited by a saw-tooth potential obtained from a Tektronix (162) waveform generator (Fig. 8). This wave form is resistively added and attenuated against a negative 170 V potential (obtained from a Tektronix 160 A power supply) so that at junction point 1 a negative saw tooth of approximately 5 to 6 V amplitude is obtained. The amplitude control R_5 applies

a selected amount of this saw tooth to the Goodmans puller via a transistor compound emitter follower (its function being similar to a vacuum tube cathode follower). Since the emitter follower responds to a negative signal, application of negative pulses of other wave forms could be employed with suitable adjustment of the reference potential. Minimum displacement time depends upon characteristics of the electromagnetic puller. Displacement of the puller is recorded by a vane interposed between a diffused light source and an International Rectifier Corp. B-10 photocell.

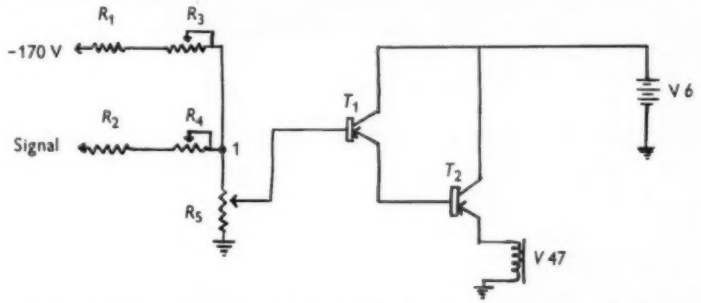

Fig. 8. Transistor control circuit for puller. R_1, R_2 = 22 kΩ; R_3, R_5 = 50 kΩ; R_4 = 1 kΩ. T_1 = Texas Instruments 2N291. T_2 = CBS 2N155. *V* 47 = Goodman's Puller.

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THE STIMULATION OF RESPIRATION BY 5-HYDROXYTRYPTAMINE IN MAN

BY VERONICA J. PARKS, A. G. SANDISON, S. L. SKINNER AND R. F. WHELAN

From the Department of Human Physiology and Pharmacology, The University of Adelaide, Australia

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5-Hydroxytryptamine has been observed to produce changes in respiration in a variety of animals, the nature of the changes displaying some species variation (Schneider & Yonkman, 1954; Westermann, 1958). In man hyperpnoea has been described following rapid intravenous injection (Page & McCubbin, 1953) although occasionally a transient apnoea precedes the hyperpnoea (Michelson, Hollander & Lowell, 1958). Continuous intravenous infusions have been observed to cause hyperpnoea (LeMessurier, Schwartz & Whelan, 1959). Evidence is presented in this paper that the respiratory changes in man result from a direct action of the drug on receptors situated on the arterial side of the systemic circulation.

METHODS

The subjects were ourselves, our colleagues and volunteer medical students. Ages ranged from 19 to 36 years. The subjects were lightly clothed and lay on a couch for at least half an hour before measurements were commenced. The room temperature was $22 \pm 1°$ C throughout. Forearm blood flow was measured by venous occlusion plethysmography with water-filled plethysmographs at a temperature of 34–35° C. A second plethysmograph applied to the other forearm was used to record pulse rate changes. Respiration rate and depth were measured with abdominal and thoracic stethographs (Dornhorst & Leathart, 1952). At the beginning of the experiment an intravenous infusion of NaCl solution 0·9 % (w/v) was begun through a needle inserted into an antecubital vein at the elbow and continued at a rate of 4 ml./min throughout by means of a mechanically driven syringe. The NaCl infusion was replaced when required by a solution of 5-hydroxytryptamine creatinine sulphate made up in saline so that the dose per minute was contained in 4 ml. The dose given was 1, 2 or 3 mg/min of the salt depending on the sensitivity of the subject (LeMessurier *et al.* 1959). The infusion lasted for one, five, ten or twenty minutes in different groups of experiments.

Oxygen consumption and carbon dioxide output were determined from the analysis (by the Henderson–Haldane technique) of expired air collected in Douglas bags before, during and after 10- or 20-min infusions of 5-hydroxytryptamine. The subjects arrived in the laboratory in the morning in the fasting state and with a minimum of previous activity. Duplicate analyses were always made. In three subjects oxygen consumption was determined by closed-circuit spirometry, the subject breathing 100 % oxygen from a spirometer throughout the experiment.

RESULTS

The pattern of the respiratory response. Intravenous infusion of 5-hydroxytryptamine in a dose of 1, 2 or 3 mg/min always resulted in an increase in the depth of respiration, commencing abruptly about 50–70 sec after the drug infusion began. Apnoea was never seen.

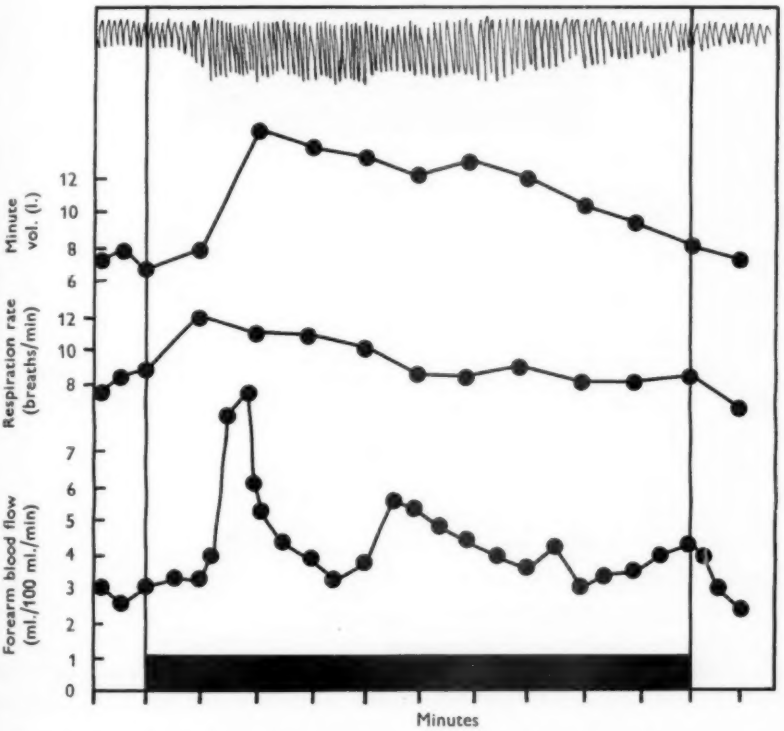

Fig. 1. A typical response of respiration and forearm blood flow to intravenous infusion of 5-hydroxytryptamine creatinine sulphate (2 mg/min) indicated by the black rectangle. The top trace is the stethograph record, inspiration downwards.

The respiration rate showed a variable change. In the majority of experiments the increase in depth of respiration was accompanied by an increase in rate, with a consequent increase in minute volume. The changes were most marked in the first 5 min of the infusion period, and the rate and depth returned to or near the resting value by the end of the 10-min infusion. Figure 1 shows a typical response of this kind, being one of twenty-two experiments on seven subjects.

In two subjects, however, the respiration rate fell with the increased depth of breathing so that the minute volume was little changed.

Time relations. Figure 2 shows the relationship of the respiratory response to the heart rate and forearm blood-flow changes in one subject. The changes in both forearm blood flow and respiration began simultaneously 60 sec after the start of the infusion of the drug and 5–10 sec

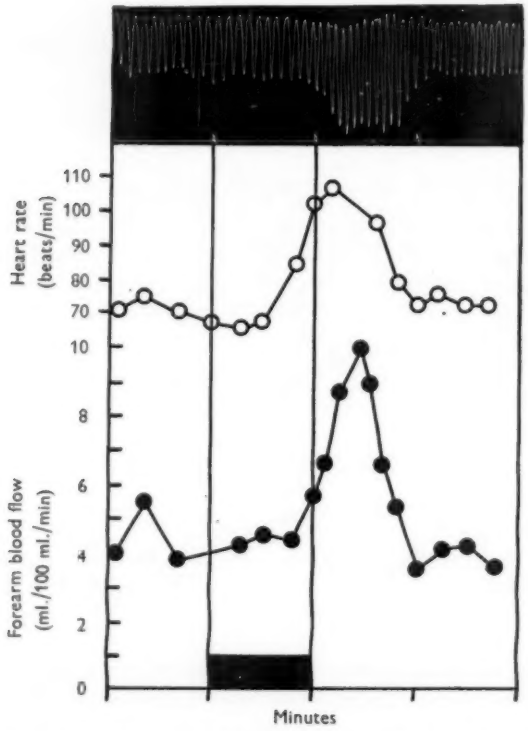

Fig. 2. The times of onset of the respiratory, cardiac and forearm flow changes following intravenous 5-hydroxytryptamine. Top trace, stethogram, inspiration downwards. The period of infusion of 5-hydroxytryptamine is indicated by the black rectangle.

after the onset of tachycardia. This relationship was seen in all of thirty-five experiments on fourteen subjects.

Oxygen consumption and carbon dioxide output. During the first 5 min of the infusion of 5-hydroxytryptamine an apparent increase in metabolic rate was indicated by an increased oxygen uptake of from 9 to 28% (Table 1). However, this was associated in each case with a more marked increase in carbon dioxide output of from 20 to 60%, indicating that the

hyperpnoea was not wholly metabolic in origin. The subject P.H., who showed least change in oxygen consumption, had only a slight increase in minute volume. During the second 5-min period oxygen consumption and carbon dioxide output were only slightly elevated above the resting levels.

TABLE 1. Oxygen consumption (ml./min, S.T.P.) during intravenous infusions of 5-hydroxytryptamine

The value for the consumption before the infusion is the mean of two consecutive 5-min samples. The values in brackets are percentages of this mean value. Infusions lasted for 5, 10 or 20 min. The post-infusion samples were collected 10–15 min after the end of the infusions.

Subject	Before	During I.V. 5-hydroxytryptamine 0–5 min	5–10 min	10–20 min	After
		Haldane analysis			
R.F.W.	166	200 (120)	—	—	160 (96)
H.M.D.	223	251 (113)	—	—	224 (100)
R.F.W.	186	238 (128)	196 (105)	—	188 (101)
S.L.S.	240	262 (109)	246 (102)	—	210 (88)
P.H.	201	206 (103)	198 (99)	—	201 (100)
A.G.S.	213	251 (118)	—	229 (107)	224 (105)
V.J.P.	149	—	—	155 (104)	131 (88)
R.F.W.	182	—	—	197 (108)	189 (104)
		Spirometric analysis			
I.S.D.	248	260 (105)	—	—	206 (83)
R.F.W.	242	251 (104)	—	—	242 (100)
N.C.T.	214	214 (100)	—	—	214 (100)

In three subjects the infusion was maintained for 20 min and the consumption during the last 10 min of this period was compared with the pre-infusion level. Under these conditions a steady state of respiration was attained and a slight increase in oxygen consumption occurred (av. 6%; Table 1). To investigate the possibility that the increases in oxygen consumption in the first 5 min of the infusions were due to oxygen storage as a result of the hyperpnoea (Murray, 1959) three experiments were carried out in which the oxygen consumption was determined spirometrically, the subjects breathing 100% oxygen for 15 min before, as well as during, 5-min infusions of 5-hydroxytryptamine. Although marked hyperpnoea occurred in each case, there were only slight increases in consumption (Table 1).

Tachyphylaxis. In ten experiments on five subjects the stimulant effect of 5-hydroxytryptamine on the respiration invariably exhibited the phenomenon of tachyphylaxis, intravenous infusions of 1 min duration having a diminished effect, or none, when repeated with an interval of 2 or 3 min to allow respiration to return to normal (Fig. 3, upper trace). Recovery of the response occurred if an interval of 20 min or more was allowed between infusions. The dilator effect of these intravenous infusions on the peripheral blood vessels did not show tachyphylaxis (Fig. 3, lower trace) nor was it seen on intra-arterial infusions in

the same subjects when the local constrictor action remained constant with repeated administrations of a range of doses from 1 to 16 μg/min (Fig. 4).

Figure 5 shows a comparison between the respiratory effects of 5-hydroxytryptamine and adrenaline. The initial effects are similar but the subsequent responses differed in that tachyphylaxis to adrenaline was not seen. The upper and lower records were continuous, with an interval of 20 min between the third and fourth injection of 5-hydroxytryptamine.

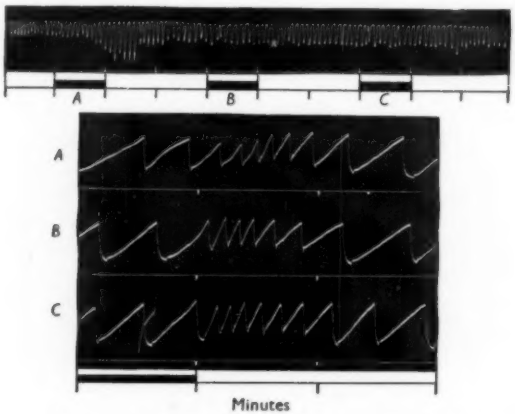

Fig. 3. Comparison of the effect of three 1-min infusions of 5-hydroxytryptamine at intervals of 2 min on the respiration (top trace) and on forearm blood flow (lower traces) measured simultaneously. The slope of each curve is directly related to the rate of blood flow. Traces *A*, *B* and *C* are continuous. The black rectangles represent the periods of infusion of 5-hydroxytryptamine.

This consistent adrenaline response was seen in all of four experiments on three subjects, infusions being repeated six times in each case. In three subjects respiratory tracings were taken during intermittent breathing of 8 % O_2 in nitrogen and 5 or 10 % CO_2 in oxygen. No tachyphylaxis of the hyperpnoeic response was seen with either of these stimuli at the same time interval as was used for repeated infusions of 5-hydroxytryptamine. The hyperpnoea was as great under these conditions as with 5-hydroxytryptamine, but less sudden in onset.

DISCUSSION

That the greater part, if not all, of the respiratory stimulation by 5-hydroxytryptamine is non-metabolic in origin is demonstrated by the abruptness of onset of the hyperpnoea within 60–70 sec of the beginning of the infusions and by the fact that carbon dioxide output invariably

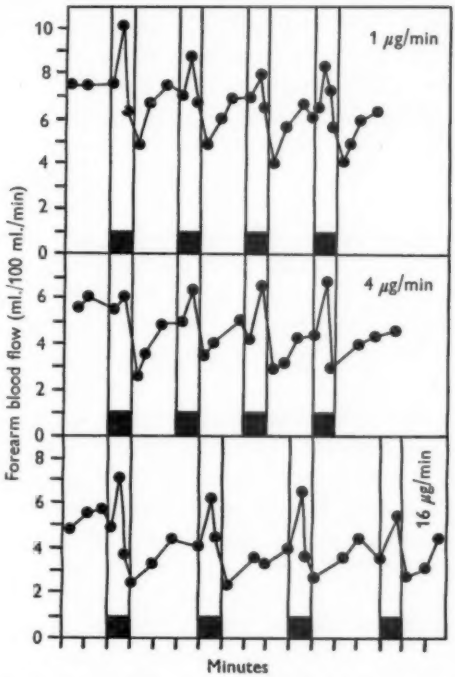

Fig. 4. Effect on forearm blood flow of repeated 1-min infusions of 5-hydroxytryptamine into the brachial artery at intervals of 2 min (1 μg and 4 μg/min) and 3 min (16 μg/min). The periods of infusion are indicated by the black rectangles.

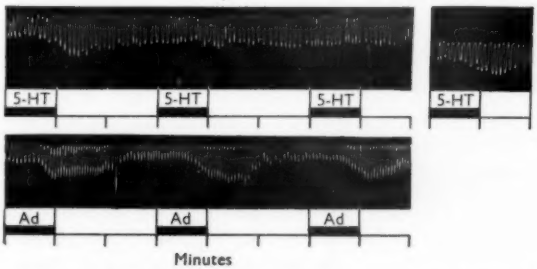

Fig. 5. Upper trace: stethograph record of responses to 1-min infusions of 5-hydroxytryptamine (1 mg/min I.V.) at intervals of 2 min. The gap in the record represents a time interval of 20 min. Inspiration downwards.

Lower trace: responses to 1-min infusions of adrenaline (10 μg/min I.V.) at intervals of 2 min. The black rectangles represent the periods of infusion of the two drugs.

exceeded the oxygen consumption. The subjects who showed the greatest increases in oxygen uptake were those who had the most pronounced increases in minute volume. Apart from a small amount of increased oxygen consumption which may be attributed to the increased activity of the respiratory muscles, most of the increase in intake can be accounted for by the phenomenon of oxygen storage as a result of the increased ventilation (Murray, 1959), since the oxygen consumption was only slightly increased in those experiments in which the subjects breathed 100% oxygen for 15 min before and during the procedure, and in whom oxygen storage might be expected to be at a minimum.

The respiratory changes were most marked in the first 5 min of the infusion period, as were the other responses recorded. These changes were not a consequence of the subjective sensations (LeMessurier *et al.* 1959) since the characteristic changes still occurred in two subjects who reported no symptoms. By the third to fifth minute of the infusion period the increases in heart rate, forearm blood flow and respiratory rate and depth had settled to sustained values less than those of the first 5 min but still above the resting level, and these values persisted throughout the remainder of the infusion. The oxygen consumption in the steady state of respiration during the second 5 min or last 10 min of the infusions showed a slight but distinct elevation above the resting value. These findings suggest that 5-hydroxytryptamine has a slight metabolic stimulant effect which, however, could not account for more than a small part of the observed increase in respiration and might well be entirely the result of increased cardiac and respiratory muscle activity. This conclusion is not in accord with the finding of Page (1957), who reports a patient in whom oxygen consumption rose from 292 to 522 ml./min (79%) in the first minute following an injection of 5-hydroxytryptamine into the heart. Although the dose of 5-hydroxytryptamine and the duration of the injection is not stated, it is probable that the oxygen storage phenomenon accounted for a good deal of this increase, since the estimation was most likely performed during the period of maximum hyperpnoea.

The possibility that the respiratory and cardiovascular effects of 5-hydroxytryptamine might be mediated by the release of adrenaline from the suprarenal glands must be considered, since Reid (1952) demonstrated such a release in the cat, and because of the similarity in man between the effects of adrenaline and 5-hydroxytryptamine on the forearm blood flow, heart rate and respiration (Whelan & Young, 1952; LeMessurier *et al.* 1959). However, repeated short infusions of 5-hydroxytryptamine elicited the phenomenon of tachyphylaxis, but similarly spaced infusions of adrenaline had no such effect. Unless it is postulated that its release from the adrenal gland exhibits tachyphylaxis, it is un-

likely that mediation by adrenaline is responsible for the respiratory response to 5-hydroxytryptamine. Furthermore, if the respiratory effect of 5-hydroxytryptamine were mediated by release of adrenaline into the blood stream an additional circulation-time delay might be expected. However, the time of onset of hyperpnoea after the beginning of infusions is the same (60–70 sec) for both adrenaline and 5-hydroxytryptamine. This time closely agrees with one circulation time, when allowance is made for time lag and dead space in the infusion device. Finally, adrenaline in an amount which produces a comparable hyperpnoea to that of 5-hydroxytryptamine causes a much greater increase in oxygen consumption (Whelan & Young, 1952).

That the tachyphylaxis of the respiratory response to 5-hydroxytryptamine represents a genuine example of this phenomenon and cannot be attributed to different plasma levels on successive infusions is indicated by the fact that the initial transient dilatation of the forearm blood vessels was comparable on each occasion, and the magnitude of these flow responses has been observed to be directly related to the dose of drug infused. That the tachyphylaxis is not due to diminished drive to respiration as a consequence of blowing off of carbon dioxide during the preceding hyperpnoea is suggested by the fact that the hyperpnoea in response to adrenaline is equally marked, but successive responses were undiminished in degree. The respiratory pattern after both drugs returned to normal between infusions, there being no tendency to apnoea as might result from excess blowing off of carbon dioxide. Furthermore, voluntary hyperpnoea to a similar degree did not diminish the response to 5-hydroxytryptamine.

The finding that the respiratory response to 5-hydroxytryptamine occurs simultaneously with the initial transient increase in forearm blood flow and 5–10 sec after the onset of the tachycardia suggests that the respiratory and forearm responses are due to arrival of the 5-hydroxytryptamine on the arterial side of the circulation, the tachycardia being a direct result of its earlier arrival in the heart. The same initial transient blood flow increase is seen in the nerve-blocked forearm when 5-hydroxytryptamine is infused intravenously (LeMessurier *et al.* 1959) and is also obtained, with a shorter latency, when comparable doses of 5-hydroxytryptamine are given by intra-arterial infusions into the arm. It is attributed therefore to a direct action of the drug on the forearm vessels. These considerations place the site of receptors for the respiratory response on the systemic arterial side of the circulation. The present results provide no evidence for any closer localization and the receptors concerned could be in the carotid or other chemoreceptor area or in the brain itself.

It has been suggested that the respiratory stimulant effect of 5-hydroxytryptamine could be a result of its vaso-spastic property causing constriction

of the vessels supplying either a chemoreceptor area or the respiratory centre, the resulting anoxia or hypercapnia providing the stimulus for hyperpnoea (Ginzel & Kottegoda, 1954; Lewis, 1958). This suggestion is supported by the finding (McCubbin, Green, Salmoiraghi & Page, 1956) that perfusion of the carotid body of the dog with 5-hydroxytryptamine increases impulse traffic in the sinus nerve to the same extent as perfusion with anoxic blood. If it is postulated that the vessels supplying the receptors concerned are constricted by intravenous 5-hydroxytryptamine it is necessary to postulate that this effect exhibits tachyphylaxis. The constrictor response of the peripheral vessels in the arm to arterial infusion, however, does not exhibit this phenomenon, although the doses used (1, 4 and 16 μg/min) cover the range of dose likely to be arriving at the respiratory receptors concerned during intravenous infusions.

It is unlikely that the tachyphylaxis was a consequence of diminishing response to local anoxia or hypercapnia due to vasconstriction in the receptor site, since no tachyphylaxis was observed in the hyperpnoeic response to breathing 8 % oxygen in nitrogen or 5 and 10 % carbon dioxide in oxygen.

The above findings lead to the conclusion that the action of 5-hydroxytryptamine on respiration represents a direct effect of the drug on receptors situated in the systemic arterial side of the circulation.

SUMMARY

1. Intravenous infusions of 5-hydroxytryptamine in man in doses of 1–3 mg/min cause a stimulation of respiration which is most marked in the first 5 min but persists in lesser degree throughout the remainder of the 10- or 20-min infusion. Tidal air always increases but respiratory rate shows a variable change. Apnoea is not seen.

2. Increased metabolic rate is not the cause of the hyperpnoea, although slight increases in oxygen consumption occur during continuous infusions. During the period of maximal hyperpnoea most of the apparent increase in oxygen consumption can be accounted for by the phenomenon of oxygen storage.

3. Tachyphylaxis of the hyperpnoea was observed on repeated infusions, but was not seen in the respiratory response to adrenaline. This fact, together with the time course of the hyperpnoea and the slight metabolic effect of 5-hydroxytryptamine, suggests that release of adrenaline is not responsible for the respiratory stimulant effect of 5-hydroxytryptamine.

4. The onset of the hyperpnoea corresponds in time with the initial effect on the forearm blood flow, indicating that 5-hydroxytryptamine stimulates receptors situated in the systemic arterial circulation.

5. Tachyphylaxis is not exhibited by the local constrictor action of 5-hydroxytryptamine on peripheral vessels.

6. These findings suggest that 5-hydroxytryptamine stimulates respiration in man by a direct action on receptors, and not indirectly as a result of vascular spasm producing anoxia of these receptors.

This work was carried out during the tenure by V.J.P. and S.L.S. of Research Fellowships of the National Health and Medical Research Council of Australia. We wish to thank our colleagues and medical students who volunteered as subjects, Mr A. McNeil and Mr R. Clark for expert technical assistance and Messrs Sandox of Basle, Switzerland, for a gift of supplies of 5-hydroxytryptamine.

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THE EFFECT OF DEOXYCORTICOSTERONE ON THE UNIDIRECTIONAL TRANSFERS OF SODIUM AND POTASSIUM INTO AND OUT OF THE DOG INTESTINE

BY E. Y. BERGER, GRACE KANZAKI AND J. M. STEELE

From the New York University Research Service Goldwater Memorial Hospital, Welfare Island, New York 17, *N.Y., U.S.A.*

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The patient with congestive heart failure or cirrhosis of the liver, who gains oedema fluid daily, fails to excrete sodium in the urine in amounts equal to dietary intake. This abnormal conservation of sodium is not limited to the kidney, since the sweat glands, salivary glands, and colons of these patients also conserve sodium (Berger & Steele, 1952). In order to effect this abnormal conservation, in the case of the nephron and colon sodium absorption is increased, while in the case of the salivary gland and sweat gland secretion is decreased.

Although this study is restricted to the intestine, it was launched with the notion that under conditions of sodium retention sodium transfer would be affected in the same manner across all four membranes. Absorption and secretion, that is, net transfer, for these four membranes may be the result of two unidirectional but opposite flows in the same limited area. It is known that this situation exists in the intestinal lumen (Visscher, Varco, Carr, Dean & Erickson, 1944; Visscher, Fetcher, Carr, Gregor, Bushey & Barker, 1944; Curran & Solomon, 1957; Berger, Kanzaki, Homer & Steele, 1959; Berger, Kanzaki & Steele, 1959). Here the addition or removal of sodium (potassium, chloride or water) is not the consequence of a simple one-way passage into or out of the lumen, but the amount appearing in the intestinal lumen is the net result of two simultaneous flows of sodium across the intestinal mucosa. Conservation of sodium may follow either a decrease in the rate at which sodium enters the lumen or an increase in the rate at which sodium leaves the lumen, or both. The object of this study was to determine which of the unidirectional flows was altered under conditions of sodium retention.

In order to determine unidirectional fluxes across a limited area of membrane, it is necessary to sample the contents of the lumen while avoiding contamination from other portions of the tubule or duct. This

poses obvious difficulties for a study of the kidney, the salivary gland or sweat gland, but segments of the intestine may easily be isolated in the dog by preparing a chronic Thiry fistula. This is an isolated segment of intestine fashioned into a pouch with an exteriorized stoma. Such Thiry fistulas were prepared in dogs. Sodium retention was induced with deoxycorticosterone acetate, since the abnormal conservation of sodium by the kidney, colon, salivary gland and sweat gland of the oedematous individual may be simulated in normal men by administration of deoxycorticosterone (Conn, 1949; Berger, Quinn & Homer, 1951; White, Entmacher, Rubin & Leiter, 1955). With the use of radio-isotopes the unidirectional fluxes of sodium and of potassium were measured across the mucosa of various segments of the dog intestine before and after the administration of deoxycorticosterone.

A study of the effects of a drug on the unidirectional fluxes of sodium across the intestine of the normal dog is complicated by a large inherent variability of repeated measurements of sodium flux under presumably the same controlled conditions (Berger, Kanzaki, Homer & Steele, 1959). In order to increase the sensitivity of the statistical evaluation of the measurements, paired experiments were employed. Immediately following 'control' measurements, deoxycorticosterone acetate was administered and a day later the measurements were repeated for the 'treated' experiment. The calculation for a significant effect was based on the difference between each 'control' and its paired 'treated' measurement.

The experiments are further hindered by another factor when deoxycorticosterone acetate is the drug under study. It is well known that the antinatriuretic effect of deoxycorticosterone is difficult to demonstrate in acute experiments on the normal dog (Barger, Berlin & Tulenko, 1958). In the experiments on the normal dog, to be described, one could not be certain that deoxycorticosterone was having an effect every time it was administered. Our solution was to accumulate a sufficient number of paired experiments to demonstrate a difference between the 'control' and 'treated' measurements.

Terminology. The terms 'unidirectional flux', 'influx' and 'efflux' deserve definition. 'Unidirectional flux' will refer to the one-way passage of electrolyte from one side of the intestinal mucosa to the other, which, in the case of sodium (and potassium) occurs concurrently with a one-way passage of sodium (or potassium) in the opposite direction. In this report 'influx' will denote flow into, and 'efflux' flow out of the intestinal lumen, and the direction of flow will be specifically stated, i.e. into or out of the lumen, unless it is otherwise obvious. The terms flux or flow will be reserved for unidirectional movements only. 'Absorption' will be used to indicate a net decrease in the amount of electrolyte or water in the lumen,

resulting from a flux out of the lumen that is greater than the flux into the lumen. Similarly, 'secretion' indicates a net increase in the amount of electrolyte in the lumen resulting from a greater flux into, than out of the lumen. Absorption and secretion will be used where unidirectional fluxes are unknown (for example, secretion of potassium by the distal portion of the nephron). 'Excretion' is reserved for the final excretory product contained in the stool.

METHODS

A chronic Thiry fistula was prepared in the dog by resecting a 20 cm section of proximal jejunum, distal ileum or distal colon with its blood supply intact, and re-establishing the continuity of the intestinal tract. One end of the resected portion was closed, and the other end was exteriorized through the skin of the abdominal wall to form a stoma through which solutions could be placed in the lumen of the isolated segment. After recovery from the operation the animals were trained to lie quietly during the experimental procedures so that anesthesia was unnecessary. The intestinal segment was flushed at the start of an experiment with a modified Ringer's solution containing (m-equiv/l.) Na 144, Cl 109, HCO_3 39, K 4, Mg 2 and SO_4 2. When the returned fluid was clear, 10 or 20 ml. of the same solution, containing in addition methyl-cellulose in concentration of 0·4 %, radiosodium (^{24}Na), and in some experiments radiopotassium (^{42}K) was pipetted into the intestinal segment through a catheter. The volume of fluid in the pouch was calculated from the amount of methyl-cellulose placed in the pouch and its concentration in subsequent samplings. In general, five or more samples were taken at 7–10 min intervals; in some experiments three samples were removed at 20 min intervals. When sampling was done in periods of 7–10 min, transfer rates were calculated for the interval between the first sample and each of the subsequent samples and the reported rate is a mean of these values. Where the samples were removed at 20 min intervals, the reported rate is a mean of the rates calculated from the first to second sample, from second to third, and from first to third.

Sodium and potassium were determined with an internal-standard flame photometer. After precipitating protein with the Somogyi zinc precipitant (Somogyi, 1930), methyl-cellulose was determined by heating 2 ml. of the supernatant fluid with 5 ml. of Harrison's diphenylamine reagent (Harrison, 1942) for 30 min at 108·3° C in an oil bath and measuring the concentration colorimetrically at 645 mμ (Kanzaki & Berger, 1959). Radiosodium (^{24}Na) was determined in the presence of radiopotassium (^{42}K) by Geiger-tube counting with and without a 350 mg/cm² aluminum filter (Tait & Williams, 1952). Radiosodium and radio-potassium were added to the modified Ringer's solution in amounts which would yield adequate counting rates and would have a ratio of sodium counts to potassium counts of 2:1 at the time of counting. Corrections were made for radioactive decay during the counting period.

Transfer rates were calculated for two compartment systems not in dynamic equilibrium (Berger & Steele, 1958). The following equations were used:

$$\beta = \frac{A - A_0}{t}\left[\frac{\ln\left[(\theta'_A - \theta'_B)/(\theta_{A_1} - \theta_{B_1})\right]}{-\ln\dfrac{[Na]^n/[MC]^n}{[Na]_1/[MC]_1}}\right] \tag{1}$$

and

$$\alpha = \frac{A - A_0}{t}\left[\frac{\ln\left[(\theta'_A - \theta'_B)/(\theta_{A_1} - \theta_{B_1})\right]}{-\ln\dfrac{[Na]^n/[MC]^n}{[Na]_1/[MC]_1}} - 1\right]. \tag{2}$$

The transfer rate of sodium (or potassium) into the bowel in micro-equivalents per minute is β, the rate out of the bowel, α. The micro-equivalents of sodium (or potassium) in the

intestine, corrected for that previously removed in sampling, is A_0 at the beginning and A at the end of a period of t min. θ_{A_1}, $[Na]_1$ and $[MC]_1$, are respectively the specific activity (counts/min/μequiv), sodium (or potassium) concentration (μequiv/ml.) and methylcellulose concentration (mg/ml.) in the first sample and θ^n_A, $[Na]^n$, and $[MC]^n$ are their values at any subsequent sampling. Plasma specific activity is denoted by θ_{B_1}, for the first and θ^n_B for any subsequent sampling. The specific activity of the plasma may be neglected for potassium ($\theta_A \gg \theta_B$) and, except where radiosodium was administered intravenously, for most experiments specific activity of plasma sodium may also be neglected (Berger, Kanzaki, Homer & Steele, 1959). Further details of the method are described in previously published work (Berger & Steele, 1958; Berger, Kanzaki, Homer & Steele, 1959; Berger, Kanzaki & Steele, 1959).

The experiments were conducted in pairs, a control experiment and one in which the drug was used. The statistical evaluation of the results was based on the difference between the experiments of each pair. Once the control experiment was completed, deoxycorticosterone acetate 2 mg/kg in sesame oil was administered intramuscularly to the dog and measurements were repeated the following day (19–21 hr after deoxycorticosterone administration). Mean values are expressed $\pm$ the standard error ($\sigma_{\bar{x}}$) where $\sigma_{\bar{x}} = \frac{\sigma}{\sqrt{N}}$ and σ, the standard deviation, $= \sqrt{\left(\left[\frac{\Sigma x^2}{N} - \bar{x}^2\right]\left[\frac{N}{N-1}\right]\right)}$. The coefficient of correlation (r) was calculated from $\sqrt{bb'}$ where b is the slope of the regression line of x on y, and b' is the slope of the regression line of y on x. The coefficient of correlation was considered significant if $3\sigma z$ did not exceed the z transformation of r.

RESULTS

Effects of deoxycorticosterone on the dog small intestine

Two animals were studied, one with a jejunal, the other with an ileal fistula. In fifteen paired experiments on these dogs, one experiment a control the other 20 hr after intramuscular administration of deoxycorticosterone, sodium transfers were not altered. In six of these pairs, where potassium transfers were measured in addition to sodium, the administration of deoxycorticosterone did not alter transfer rates of potassium (Table 1).

Effects of deoxycorticosterone on the dog large intestine

Absorption and secretion. Four animals were studied, each with a Thiry fistula prepared from the colon. Twenty-nine pairs of experiments were conducted where sodium transfer was measured, and in thirteen of these potassium was measured in addition. Deoxycorticosterone increases the absorption of sodium from the colon and increases the secretion of potassium into the colon (Table 2). Those experiments which had the largest absorption of sodium from the lumen were not the same experiments as those which had the largest secretion of potassium (the correlation coefficient for net sodium difference between control and treated against net potassium difference was $+0{\cdot}48$).

Unidirectional fluxes. Sodium decreased in the colonic lumen in consequence of an increase in the rate of transfer out of the colon; whereas the

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TABLE 1. The effect of deoxycorticosterone on the unidirectional fluxes of sodium and potassium across the dog small intestine
(μequiv/min/20 cm length of intestine)

Date of control measurement	Sodium									Potassium								
	Rate into lumen			Rate out of lumen			Net change in lumen			Rate into lumen			Rate out of lumen			Net change in lumen		
	CONT*	DOCA†	R_x–C‡	CONT	DOCA	R_x–C	CONT	DOCA	R_x–C	CONT	DOCA	R_x–C	CONT	DOCA	R_x–C	CONT	DOCA	R_x–C
Dog MAR, jejunum operation 23. x. 52																		
3. ii. 53§	65·4	84·0	+18·6	79·9	94·8	+14·9	−14·5	−10·8	+3·7									
10. iii. 53	78·4	113·9	+35·5	101·4	110·3	+8·9	−23·0	+3·6	+26·6									
7. iv. 53§	70·0	93·3	+23·3	62·9	99·7	+36·8	+7·1	−6·4	−13·5									
16. x. 56	65·5	83·2	+17·7	56·9	66·8	+9·9	+8·6	+16·4	+7·8									
30. x. 56	66·6	105·2	+38·6	55·3	77·4	+22·1	+11·3	+27·8	+16·5									
7. v. 57	80·5	64·7	−15·8	46·9	42·6	−4·3	+33·6	+22·1	−11·5	2·80	2·05	−0·75	1·59	1·25	−0·34	+1·21	+0·80	−0·41
30. vii. 57	71·4	70·7	−0·7	65·8	60·4	−5·4	+5·6	+10·3	+4·7	5·62	2·21	−3·41	5·24	1·89	−3·35	+0·38	+0·32	−0·06
Dog BRN, ileum operation 6. vi. 52																		
21. x. 52**	81·7	105·4	+23·7	95·4	89·2	−6·2	−13·7	+16·2	+29·9									
17. iii. 53	72·9	81·1	+8·2	65·3	67·7	+2·4	+7·6	+13·4	+5·8									
14. iv. 53§	79·9	78·1	−1·8	80·3	60·7	−19·6	−0·4	+17·4	+17·8									
16. x. 56	52·8	59·2	+6·4	44·7	51·9	+7·2	+8·1	+7·3	−0·8									
18. vi. 57	98·2	82·1	−16·1	42·3	30·8	−11·5	+55·9	+51·3	−4·6	2·66	3·87	+1·21	1·05	1·67	+0·62	+1·61	+2·20	+0·59
23. vii. 57	82·1	74·6	−7·5	49·7	52·3	+2·6	+32·4	+22·3	−10·1	2·98	2·84	−0·14	1·78	1·60	−0·18	+1·20	+1·24	+0·04
6. viii. 57	94·3	70·0	−24·3	32·8	39·2	+6·4	+61·5	+30·8	−30·7	3·65	2·75	−0·90	1·53	1·78	+0·25	+2·12	+0·97	−1·15
3. xii. 57	51·0	75·4	+24·4	40·6	52·3	+11·7	+10·4	+23·1	+12·7	1·88	2·41	+0·53	1·71	1·88	+0·17	+0·17	+0·53	+0·36
Mean	74·0	82·7	+8·7	61·3	66·4	+5·1	+12·7	+16·3	+3·6	3·26	2·69	−0·58	2·15	1·68	−0·47	+1·12	+1·01	−0·10
σ	13·2	15·7		20·3	23·5		24·1	15·2		1·29	0·65		1·54	0·24		0·74	0·67	
$\sigma_{\bar{x}}$	3·4	4·0	5·0	5·2	6·1	3·6	6·2	3·9	4·2	0·52	0·27	0·65	0·63	0·10	0·59	0·30	0·27	0·25
t			1·7			1·4			0·9			0·88			0·80			0·42

* CONT control measurements; † DOCA measurements ~ 20 hr after an intramuscular injection of deoxycorticosterone acetate in sesame oil, 2·0 mg/kg body weight; ‡ R_x–C change in flux rate following deoxycorticosterone; § Dog on high-salt diet by adding salt to drinking water (150–250 m-equiv/l.). Usual diet contained up to 6 m-equiv kg body weight/day; the high-salt diet 7–15 m-equiv/kg/day; ** Measurements made with radiosodium administered intravenously.

TABLE 2. The effect of deoxycorticosterone on the unidirectional fluxes of sodium and potassium across the dog large intestine
(μequiv/min/20 cm length of intestine)

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Date of control measurement	Sodium									Potassium								
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	CONT*	DOCA†	R_x–C‡	CONT	DOCA	R_x–C	CONT	DOCA	R_x–C	CONT	DOCA	R_x–C	CONT	DOCA	R_x–C	CONT	DOCA	R_x–C
Dog JIT, colon operation 7. ii. 57																		
2. iv. 57	22·0	16·2	−5·8	68·0	52·4	−15·6	−46·0	−36·2	+9·8	4·18	18·47	+14·29	2·95	6·73	+3·78	+1·23	+11·74	+10·51
11. iii. 58	31·4	17·3	−14·1	63·5	50·9	−12·6	−32·1	−33·6	−1·5	4·48	4·05	−0·43	1·16	1·10	−0·06	+3·32	+2·95	−0·37
Dog MAM, colon operation 27. ii. 57																		
9. iv. 57	21·3	25·4	+4·1	73·9	75·0	+1·1	−52·6	−49·6	+3·0	5·61	7·04	+1·43	4·34	3·82	−0·52	+1·27	+3·22	+1·95
6. viii. 57	22·0	23·2	+1·2	70·1	86·8	+16·7	−48·1	−63·6	−15·5	2·05	7·89	+5·84	2·30	5·34	+3·04	−0·25	+2·55	+2·80
11. iii. 58	26·7	33·9	+7·2	64·5	69·8	+5·3	−37·8	−35·9	+1·9	6·19	8·31	+2·12	3·72	5·01	+1·29	+2·47	+3·30	+0·83
8. vii. 58	29·0	33·6	+4·6	66·1	89·1	+23·0	−37·1	−55·5	−18·4	5·68	6·95	+1·27	3·01	4·37	+1·36	+2·67	+2·58	−0·09
Dog LDY, colon operation 2. iv. 52																		
5. xi. 52§	27·2	37·2	+10·0	75·3	85·7	+10·4	−48·1	−48·5	−0·4									
27. i. 53§	39·2	24·1	−15·1	57·6	64·5	+6·9	−18·4	−40·4	−22·0									
17. ii. 53§	21·4	25·4	+4·0	50·0	61·1	+11·1	−28·6	−35·7	−7·1									
3. iii. 53	29·8	33·8	+4·0	72·5	88·9	+16·4	−42·7	−55·1	−12·4									
31. iii. 53§	30·1	22·9	−7·2	65·8	52·1	−13·7	−35·7	−29·2	+6·5									
9. x. 56	17·7	16·9	−0·8	45·0	44·9	−0·1	−27·3	−28·0	−0·7									
30. x. 56	26·7	39·8	+13·1	48·3	59·6	+11·3	−21·6	−19·8	+1·8									
13. xi. 56	22·6	12·6	−10·0	49·6	37·8	−11·8	−27·0	−25·2	+1·8									
23. iv. 57	26·5	20·7	−5·8	44·5	50·7	+6·2	−18·0	−30·0	−12·0	0·78	3·58	+2·80	0·96	1·32	+0·36	−0·18	+2·26	+2·44
Dog SKP, colon operation 4. vi. 52																		
15. x. 52	31·1	29·4	−1·7	53·3	96·7	+43·4	−22·2	−67·3	−45·1									
28. x. 52§	63·0	17·4	−45·6	35·8	75·4	+39·6	+27·2	−58·0	−85·2									
5. xi. 52§	16·4	22·6	+6·2	55·8	75·0	+19·2	−39·4	−52·4	−13·0									
10. ii. 53§	28·4	45·6	+17·2	69·4	103·5	+34·1	−41·0	−57·9	−16·9									
25. ii. 53	53·5	39·3	−14·2	70·6	80·5	+9·9	−17·1	−41·2	−24·1									
24. iii. 53	28·3	36·8	+8·5	51·6	79·5	+27·9	−23·3	−42·7	−19·4									
28. iv. 53§	22·1	37·9	+15·8	55·4	85·5	+30·1	−33·3	−47·6	−14·3									
9. x. 56	17·0	19·3	+2·3	45·3	49·1	+3·8	−28·3	−29·8	−1·5									
22. i. 57	15·2	32·4	+17·2	43·7	64·8	+21·1	−28·5	−32·4	−3·9	2·21	6·12	+3·91	1·83	2·50	+0·67	+0·38	+3·62	+3·24
5. ii. 57	22·6	12·7	−9·9	50·7	44·4	−6·3	−28·1	−31·7	−3·6	1·84	4·40	+2·56	1·80	2·05	+0·25	+0·04	+2·35	+2·31
16. iv. 57	24·0	22·9	−1·1	58·8	54·3	−4·5	−34·8	−31·4	+3·4	1·41	4·08	+2·67	1·42	1·73	+0·31	−0·01	+2·35	+2·36
30. vii. 57	23·0	15·1	−7·9	39·0	53·8	+14·8	−16·0	−38·7	−22·7	1·51	4·72	+3·21	0·92	2·65	+1·73	+0·59	+2·07	+1·48
8. vii. 58	44·6	21·6	−23·0	28·6	60·9	+32·3	+16·0	−39·3	−55·3	4·85	4·35	−0·50	0·72	1·63	+0·91	+4·13	+2·72	−1·41
15. vii. 58	29·3	35·5	+6·2	27·1	41·5	+14·4	+2·2	−6·0	−8·2	3·02	13·26	+10·24	1·11	2·68	+1·57	+1·91	+10·58	+8·67
Mean	28·0	26·6	−1·4	55·2	66·7	+11·5	−27·2	−40·1	−12·9	3·37	7·17	+3·80	2·02	3·15	+1·13	+1·35	+4·02	+2·67
σ	10·6	9·2		13·4	18·0		17·9	13·8		1·87	4·30		1·16	1·76		1·44	3·21	
$\sigma_{\bar{x}}$	2·0	1·7	2·5	2·5	3·3	3·0	3·3	2·6	3·7	0·52	1·19	1·16	0·32	0·49	0·34	0·40	0·89	0·94
t			0·6			3·9			3·5			3·27			3·35			2·85

* Abbreviations as for Table 1.

mean control value was $55{\cdot}2 \pm 2{\cdot}5$ μequiv/min, after deoxycorticosterone it increased to $66{\cdot}7 \pm 3{\cdot}3$ μequiv/min. Sodium transfer into the colonic lumen was unaffected by deoxycorticosterone, $28{\cdot}0 \pm 2{\cdot}0$ in the control as compared with $26{\cdot}6 \pm 1{\cdot}7$ μequiv/min after deoxycorticosterone.

Potassium transfer rates into and out of the colonic lumen were both increased by deoxycorticosterone; the influx into the lumen increased to a greater degree than the efflux, with a resultant net increase in the potassium in the lumen. The mean potassium influx into the lumen was $3{\cdot}37 \pm 0{\cdot}52$ in the control experiments against $7{\cdot}17 \pm 1{\cdot}19$ μequiv/min after deoxycorticosterone. The mean potassium efflux from the colonic lumen was $2{\cdot}02 \pm 0{\cdot}32$ in the control and $3{\cdot}15 \pm 0{\cdot}49$ μequiv/min after deoxycorticosterone.

Those experiments where sodium efflux from the colonic lumen was largest were not the same experiments as those where potassium influx was largest ($r = +0{\cdot}18$) neither was the potassium efflux correlated with sodium influx ($r = -0{\cdot}07$).

DISCUSSION

Intestinal absorption and secretion of sodium and potassium

Small intestine. The data fail to demonstrate an effect of intramuscularly administered deoxycorticosterone acetate on the absorption or secretion of sodium or potassium by the small intestine. However, by other techniques, possible hormonal influences on the small intestine have been shown. Field, Swell, Dailey, Trout & Boyd (1955) have demonstrated that the sodium concentration decreased and potassium concentration increased in the terminal ileum of the dog when the daily dietary sodium was changed from 85 to 1 μequiv. Clark (1939) found that following the administration of sodium chloride by mouth, more chloride was found in the intestine of the adrenalectomized rat than in the normal control. Dennis & Wood (1940), observing two adrenalectomized dogs, found a decrease in the absorption of sodium, potassium and chloride from chronic lower ileal Thiry fistulas when adrenal cortical extract was withheld. The difference between the present observations and those of Clark (1939), Dennis & Wood (1940) and Field *et al.* (1955) is in all probability a consequence of the type of experiment performed.

Large intestine. There is a considerable body of evidence to indicate that the large intestine, as opposed to the small intestine, does alter the absorption or secretion of electrolyte in response to hormonal factors. The administration of deoxycorticosterone to the rat decreases faecal sodium excretion (Berger *et al.* 1951). The administration of 9-α-fluorohydrocortisone to the dog decreases faecal sodium and increases faecal potassium (Poutsiaka, Thomas & Linegar, 1957). In the dog, during the development

of ascites following constriction of the thoracic inferior vena cava, there is a decrease in faecal sodium and an increase in faecal potassium (Davis & Howell, 1953). This reduced ratio of sodium:potassium in the faeces returns toward normal after bilateral adrenalectomy (Davis, Howell & Southworth, 1953) and the reduced ratio has been correlated with increased aldosterone excretion in the urine (Davis, Ball, Bahn & Goodkind, 1959).

In man on a high-salt diet, measurements suggest that the administration of deoxycorticosterone decreases stool sodium and increases stool potassium (Relman & Schwartz, 1952), whereas on a normal salt intake, and receiving cation exchange resin by mouth, the administration of deoxycorticosterone acetate reduces the amount of sodium appearing with the resin in the stool (Berger *et al.* 1951). More sodium is removed by the resin from the normal individual than from the oedematous patient, who fails to excrete sodium in the urine in amounts equal to the dietary intake (Berger & Steele, 1952). Administration of resin to individuals who lack the sodium-retaining hormone (e.g. in Addison's disease or after bilateral adrenalectomy) results in a large excretion of sodium in the stool, which again may be reduced by the administration of deoxycorticosterone (Emerson, Kahn & Jenkins, 1953). Duncan, Liddle & Bartter (1956) demonstrated that when large amounts of sodium were removed in the stool by resin, the urinary aldosterone was diminished, and conversely the faecal excretion of small amounts of sodium was associated with large amounts of urinary aldosterone. The evidence cited indicates that the faecal excretion of sodium in rat, dog and man is in part regulated by the presence or absence of mineralocorticoids.

Unidirectional fluxes of sodium and potassium

Small intestine. Turning our attention to the unidirectional fluxes which determine absorption or secretion of sodium or potassium, these fluxes across the small intestine were not altered by deoxycorticosterone. Similarly, Barnett, Turner & Hardy (1958) found that when radiosodium was placed in a chronic Thiry ileal loop, the subsequent blood levels of radiosodium were the same in six dogs, four serving as controls and two receiving 20 mg of ACTH intravenously.

Large intestine. As would be expected from the observations of faecal electrolyte excretion, there were changes in the unidirectional transfer rates of sodium and potassium across the colon after deoxycorticosterone. Sodium transfer into the lumen of the colon was unchanged, while transfer out of the lumen was increased. The unidirectional potassium flux into the colon increased after deoxycorticosterone to a greater extent than the increase in potassium flux out of the colon, with a resultant net increase in the amount of potassium in the colon lumen. Although the efflux of

sodium from the colonic lumen and the influx of potassium into the lumen are increased after deoxycorticosterone, the experiments with the largest sodium efflux were not the same experiments as those with the largest potassium influx ($r = +0{\cdot}18$). The data do not support an exchange of sodium for potassium across the intestinal mucosa after deoxycorticosterone, such as has been considered (from observations of the effects of acetazoleamide and mercury) to occur across the distal portion of the kidney tubule (Berliner, Kennedy & Orloff, 1951; Mudge, 1953).

Sweat gland, salivary gland and kidney

From necessarily indirect evidence, sodium is considered to be secreted by the proximal portion of the sweat gland, parotid gland and submaxillary gland and then reabsorbed distally (White *et al.* 1955; Schwartz & Thaysen, 1956). If deoxycorticosterone has an effect on the sweat and salivary glands similar to that on the intestine, where it increases the rate of sodium transfer from lumen to blood, then a movement of sodium from lumen to blood exists in the sweat and salivary glands and this would add further indirect evidence that sodium absorption occurs in these glands.

As yet little can be said about the existence of two opposite unidirectional fluxes occurring proximally and again distally across the sweat and salivary glands. With regard to the kidney, Solomon, Hanenson, Shipp, Windhager & Schatzmann (1957) and Oken, Whittembury, Windhager, Schatzmann & Solomon (1959) have reported unidirectional movements of sodium into and out of the proximal tubule of Necturus kidney. Tosteson & White (1959), using tritiated water and stop-flow urine samples, have indicated that in the dog kidney water moves into and out of both the proximal and distal tubules. The intestine allows for direct measurement of such unidirectional flows occurring within an area of histologically similar intestinal mucosa and permits the *in vivo* study of the effect of various drugs on such fluxes.

SUMMARY

1. Measurements of the unidirectional flux rates of sodium and potassium across the dog colon demonstrated the manner in which deoxycorticosterone increased sodium absorption and potassium secretion.

2. Deoxycorticosterone increased the rate at which sodium left the colon, with little effect on the influx of sodium into the colon.

3. In the case of potassium, deoxycorticosterone increased potassium flux both into and out of the intestine; the rate of potassium influx into the colon increased to a greater degree than that of efflux, resulting in a net increase of potassium in the colon.

4. In contrast to the colon, no effect of deoxycorticosterone on the transfer rates of sodium or potassium across the dog small intestine was demonstrated.

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IS MUSCLE CONTRACTION INITIATED BY INTERNAL CURRENT FLOW?

By O. STEN-KNUDSEN

From the Institute of Neurophysiology, University of Copenhagen, Denmark

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It is generally agreed that of the series of events causing contraction in a muscle fibre the first is a reduction of the potential difference across the membrane ('depolarization'). Regarding the succeeding processes which lead eventually to activation of the contractile proteins little is known at present. These processes do not seem to involve inward diffusion of a substance liberated at the surface (Hill, 1948), and some other mechanism must be looked for by which the influence of a potential change across the membrane is conveyed inwards over a considerable distance (more than 50 μ). Quite recently a clue has been provided by experiments of Huxley & Taylor (1958) which strongly suggest that in frogs the disturbance is conducted inwards along a structure adjacent to the Z membrane.

During the passage of an action potential a transient electric current of considerable magnitude flows longitudinally through the interior of the muscle fibre. To account for the rapid onset of contraction it has sometimes been suggested that this current itself, and not the change of the membrane potential, is the causative factor setting off contraction. An elaborate treatment of this hypothesis has been given by Bay, Goodall & Szent-Györgyi (1953), who gave the moving internal electric field the name 'window field'. In spite of its attractive simplicity, this theory has generally received little support. In the first place the theory is only applicable to a limited extent, since in many muscle fibres (slow fibres of frog and many arthropod muscles) a nerve stimulus does not give rise to a propagated action potential, but only to a depolarization which spreads out passively for a short distance from each terminal region. Nevertheless, a nerve impulse causes the fibre to contract because numerous nerve terminals are distributed along the fibre surface, which in this way is depolarized everywhere almost simultaneously. Secondly, in twitch muscle fibres where the theory should apply it has been shown that, in the absence of propagation, muscles exposed to a longitudinal homogeneous electric field contract only in the terminal portion of the cathodic region, where the membrane potential is reduced, while no contraction occurs in the mid region, where the current density inside the

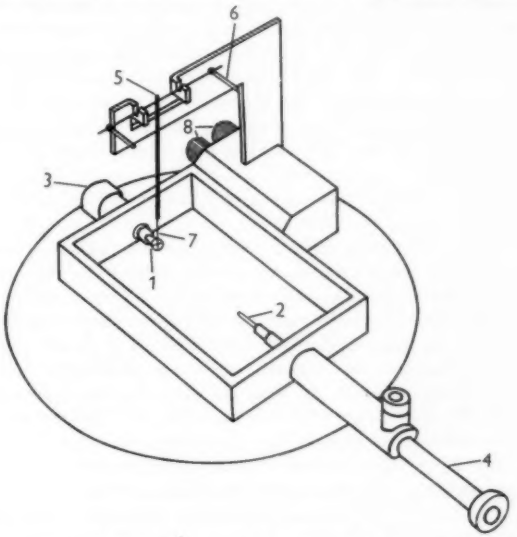
fibre is at a maximum, or at the anodic region (Sten-Knudsen, 1954). Moreover, stimulation of a non-propagating muscle with a transverse a.c. field causes the muscle to contract along its whole length and to produce almost the whole tetanus tension (Katz & Lou, 1947; Sten-Knudsen, 1954). In this case the internal longitudinal current flow is negligible.

Recently Csapo & Suzuki (1958) have claimed that they have restored the experimental foundation for the 'window field' theory. Their evidence is based upon three types of observation on the sartorius muscle, which consists of fast striated fibres arranged in a longitudinal but not strictly parallel manner. (*a*) When a muscle is suddenly exposed to a strong KCl solution (20 mM-K or more), it goes into a permanently contracted state (contracture), but the tension produced is only a small fraction of the normal tetanus tension. (*b*) When the KCl concentration is reduced to 16 mM-K, i.e. slightly less than would produce contracture and considerably more than is necessary to block propagation of the action potential, a strong longitudinal a.c. field (6–20 V/cm) causes contraction along the whole length of the muscle. Csapo & Suzuki observed that the shortening was greater in the middle portion than at the ends, and at threshold strength (4 V/cm) only the middle portion shortened. (*c*) The high-frequency transverse field is only effective within a very narrow range of K concentrations above the minimum blocking concentration. From these observations Csapo & Suzuki concluded that the longitudinal internal current is more directly linked to contraction than depolarization, but the latter is an essential 'priming' step which must precede or coincide with an effective current flow.

This hypothesis presented an attempt to reinterpret part of the evidence brought forward against the original 'window field' theory. If true, it would, in spite of its complicated mechanism, have an important bearing upon the problem of excitation–contraction coupling, as is indicated in a recent review (Gelfan, 1958). For these reasons it was thought desirable to repeat Csapo & Suzuki's experiments. To avoid the complications introduced by using a whole muscle with the fibres not all running in the same direction, the present study was performed on single isolated fibres. It will be shown that there is a distinct discrepancy between Csapo & Suzuki's observations and my own, and the reasons for this have been elucidated. Neither the experimental findings nor the theoretical treatment lend any support to the 'window field' theory in its old or new form. A preliminary report of some of the results was made at the Second Conference on Muscular Contraction held by the New York Academy of Sciences (Buchthal & Sten-Knudsen, 1959).

METHODS

Preparation. Single fibres from the semitendinosus muscle of German brown frogs (*Rana temporaria*) were isolated at room temperature in a Ringer's solution of the following composition (mM): NaCl 116, KCl 2, $CaCl_2$ 1·8. A small bundle of 20–50 fibres, with free tendinous parts 2–3 mm long at either end, was transferred to the experimental Perspex trough in which the final isolation took place (Text-fig. 1). The two tendons were clamped by the two pairs of Perspex forceps (1) and (2) and rotated by them (3) and (4) so as to expose the most convenient places for the separation of the fibres. It is important for the survival of the fibres to cut rather than to tear the connective tissue which holds them together. When the fibres are held firmly together it is an advantage to 'cut' the connective tissue by means of a broken micro-electrode fixed to a preparation needle.



Text-fig. 1. Combined dissection and experimental Perspex trough for longitudinal and transverse a.c. stimulation of single muscle fibres. The tendon of a small fibre bundle is clamped to the pair of forceps (1) and (2) and rotated by them (3) and (4) to facilitate isolation of a fibre. For isotonic recording the lever axis (5) is placed in the groove of the gallows and one tendon end is pierced by the lever needle (7) and cut free from (1), the load on the fibre depending on the length of the counterweights (6). The final adjustments are made by the set screws (8) and the bar (4) of the pair of forceps (2). For isometric recording the lever is replaced by an electromechanical transducer. For longitudinal or transverse stimulation platinum plate electrodes are placed at either end or side walls.

Marking and photography. Small graphite particles, 20–30 in number, were placed upon the upper surface of the fibre so that length changes could be watched and photographed. At the terminal portions of the fibre marks were placed at intervals of 0·5–1 mm. Once these particles are in position they stick firmly to the surface and move with it. The whole length of the fibre was photographed with transmitted light on 9 × 12 cm plates (fine-grain, Isopan F) with an ordinary photographic objective (Sonnar 1:1·5, focal length 5 cm, exposure time 1/25–1/50 sec). The primary magnification was 4·5 times.

Mounting. The one tendon end of the fibre was fixed to the pair of forceps (2) while the other was fixed to the isotonic lever (5) of aluminium tube, 44 mm long and 0·6 mm in diameter. The torque produced by the counterweight (6) fixed on the lever axis was adjusted so that the fibre was loaded with about 12 dynes when fixed to the lever needle (7). The position of the lever was adjusted by screws (8) and the tendon was then pierced by the lever needle and cut free from the left pair of forceps (1). Finally, the fibre was adjusted perpendicularly to the end walls of the trough and the right pair of forceps (4) was pulled out to make a free distance of 3–4 mm between the needle and the left pair of forceps. For isometric recording the lever was replaced by an electromechanical transducer (R.C.A. 5734), one tendon end being tied to the elongated pin of the transducer.

Stimulation. The electrodes consisted of thin platinum plates affixed to Perspex plates 2 mm thick. For longitudinal stimulation plates covering the end walls of the trough were placed perpendicularly to the fibre axis, each plate being provided with a small slit for the forceps. Since the distance from each plate to the terminal portions of the fibre was more than five times the width of the slit, any non-homogeneity of the field around the fibre caused by the slit could be neglected. For transverse stimulation two platinum plates 6 cm long placed parallel to the fibre were used. The electrodes were connected to the output of an a.c. power amplifier with adjustable output impedance, providing an output of 15 W with a total distortion less than 0·1 %. In the bath this corresponded to a longitudinal a.c. field of 7·4 V/cm, r.m.s. The power amplifier was driven by a sine wave generator and the a.c. voltage across the electrodes was measured by recording the two-way rectified and filtered output from the power amplifier.

The fibre was tested for its ability to give propagated twitches and, if found satisfactory, left for more than an hour. Only fibres giving proper twitches after this time were used for the experiment. The muscle impulses were blocked by adding a sufficient amount of a 120 mM-KCl stock solution to raise the concentration in the Ringer's solution to 14–16 mM (i.e. 7–8 times the normal value). After the experiment the K concentration of the excess K Ringer's solution was determined by means of a flame photometer.

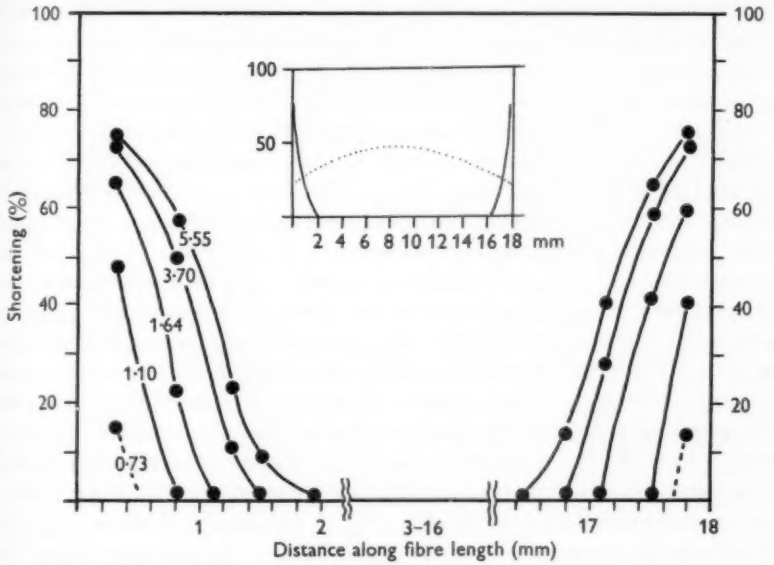
General procedure. When blocked, the fibre was photographed at rest and during longitudinal a.c. stimulation, about 2 sec after the beginning of the stimulation. The fibre was observed through a dissecting microscope throughout this procedure to be certain that the contracted state of the fibre was constant during the stimulation period. At intervals of 5 min, the field strength was altered and the fibre again photographed at rest and during a.c. stimulation. The temperature was raised by less than 0·5° C with maximum power delivered to the bath for 2 sec.

RESULTS

The localization of contraction in the longitudinal a.c. field

Contraction with reversible shortening and relaxation. An example is shown in Pl. 1. The fibre was 18 mm long; it was soaked in 14 mM-K Ringer's solution and the time required to produce the block was about 2 min. The whole length of the fibre marked with graphite particles is shown on the left-hand side of Pl. 1, both at rest (*r*) and during stimulation (*c*) with a longitudinal a.c. field (100 c/s) at about 5 V/cm, r.m.s. strength, the two frames being aligned with respect to a mark in the mid region of the fibre. The effect of the field is to cause an average shortening of about 8 % but with contraction entirely localized to the end regions. The main part of Pl. 1 shows enlargements of the end portions of the fibre with all frames aligned with respect to one mark placed at a distance of about

5 mm from the ends. The upper row of each set shows the resting fibre, and those below show the effect of the longitudinal field (100 c/s). With a field strength of 0·73 V/cm, r.m.s. (threshold) a shortening was just barely visible at the most terminal portions of the fibre, as estimated by making use of 'natural' marks on the fibre, and extended a distance of less than 0·2 mm from the tendon. With increasing field strengths shortening increased strongly and at 5·55 V/cm the portions nearest to the tendons had



Text-fig. 2. To show the extent and degree of shortening along the fibre during longitudinal a.c. stimulation (100 c/s), the field strength (V/cm, r.m.s.) is indicated by the numbers on the curves. Ordinate, relative shortening of each length element; abscissa, distance along the length of the fibre, with the mid region strongly compressed. In the inset diagram is shown the distribution of shortening along the whole fibre (full line); dotted line, an example of Csapo & Suzuki's (1958, Fig. 3, 8 V/cm, 60 c/s) results.

shortened by almost 80 %. However, the total average shortening of the fibre was only 8 % (0·7–0·8 mm of each end region in a fibre of 18 mm length). The effect of the field on the extent of contraction and the degree of shortening along the fibre is shown in Text-fig. 2. The relative shortening of each length element, $(L_r - L_c)/L_r$, where L_r and L_c are the length of each element in the resting and stimulated state, is plotted against its position in the resting fibre; the numbers on the curves indicate the value of each field strength. With increasing field strength both the degree and the extent of contraction increased. Thus, at 3·7 V/cm maximum shortening was almost 80 %. However, this extreme degree of shortening was

confined to about the 0·2 mm next to the tendon, and the strength of the contraction decreased steeply with the distance from the end of the fibre; the whole extent of the contracted region comprising only 1·5 mm of each fibre end. An increase in the field strength to 5·55 V/cm resulted in an over-all increase in the extent of contraction by 0·2 mm, while the maximum shortening only increased very little. Thus, even at such high field strengths contraction only extended over less than 2 mm at the end regions, while the remaining 14 mm length of the fibre (80 %) showed no sign whatsoever of contraction. In the inset diagram of Text-fig. 2 is shown the extreme concave shape of the graph in contrast to the convex curves presented by Csapo & Suzuki (1958), one of which for the sake of comparison is indicated by the dotted line in the diagram.

With field strengths up to 5 V/cm (100 c/s) the fibre returned to its original length after each stimulation. Therefore, to present the data in the least complicated manner the photographs in the resting state taken before each stimulation are not shown in Pl. 1.

Irreversible effects of too high field strengths. With field strengths above 5 V/cm (100 c/s) the end portions of the fibre never completely returned to their initial relaxed state, indicating that the longitudinal field at these intensities also produced some permanent after-effects in the fibre. In addition to the extreme shortening of the end regions of the fibre, field strengths well above 5 V/cm also caused the fibre to shorten in its whole length. Both effects are illustrated in Pl. 2. The row *r* of the upper set of photographs shows the resting fibre. It was photographed 5 min after a 2 sec stimulation at 5·55 V/cm (100 c/s). A shortening of about 10 % was still present in the regions occupying the 0·2–0·3 mm next to the tendons (cf. Pl. 1). The second row shows the effect of a longitudinal field of 5·8 V/cm strength. In the first phase of stimulation contraction was as usual confined only to the ends of the fibre. After about 1 sec stimulation a new region of contraction, appearing as a local bulge (indicated by the arrow), was created next to the extremely contracted terminal portions. This locally shortened region moved along the fibre and vanished after travelling a few millimetres, to be followed by a new 'wave' originating from the same place. During this time the lever end showed small irregular movements and after about 3 sec of stimulation the fibre had shortened further by a few millimetres, the ends, however, remaining extremely shortened. When the field was increased well above this 'threshold', e.g. to 7·3 V/cm, the pattern of contraction changed. In addition to the extreme shortening at the ends the extent of contraction suddenly spread rapidly from both end regions towards the middle of the fibre, which ended up with an average total shortening of about 40 %. However, this type of contraction only slightly resembled a normal isotonic tetanus. It differed in so far as

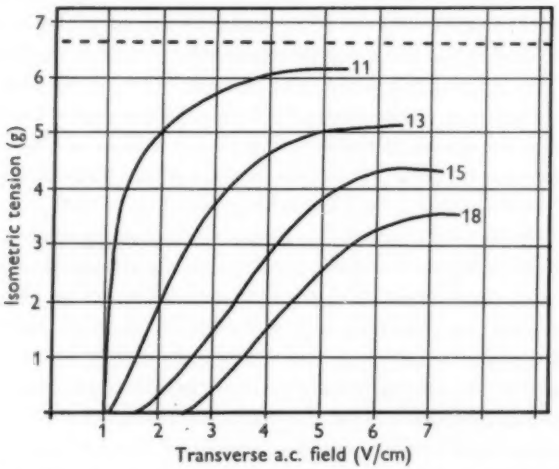
'waves' of contraction travelled along the whole length of the fibre so that local contractions and relaxations occurred alternately down the length of the fibre, with the result that the lever end moved irregularly with an amplitude of several millimetres. Moreover, the strongest contraction was confined to the end regions, the maximum shortening here being almost 80 % in contrast to the average shortening of about 40 % of the whole fibre. The next two photographs (7·3 V/cm on Pl. 2) were taken after the over-all shortening had occurred and show the difference in thickness and relative shortening along the length of the fibre, especially at the terminal portions of both ends. To illustrate the after-effects of strong stimulation, the rows *b* and *b'* in the lower set in Pl. 2 show the fibre photographed 4 sec after the termination of stimulation with an a.c. field of 7·3 V/cm applied for 3 sec. For the sake of comparison the initial fibre length is shown in rows *a* and *a'*. A partial contracture of more than 50 % shortening remained at the end regions for a distance of about 1 mm measured from the tendons, and only a very slight change in this state was observed 5 min later. The field strength ('threshold') which was just strong enough to set off these wave-like contractions gradually increased with repeated stimulations. After about 10 stimulations the fibre gave no response, even at the highest field strength available. Thus, there seems to be no doubt that longitudinal a.c. stimulation at high field strengths produced irreversible changes in the fibre, presumably by damaging the fibre membrane at the end regions where the membrane is subjected to an excessive potential difference. It is then observed that a slight misalignment of the fibre with respect to the direction of the field will temporarily restore its responsiveness, presumably because the current can then enter a region where the membrane is still undamaged.

The response of the muscle fibre just described occurred not only with 14 mM-K Ringer's solution and at 100 c/s. It was also observed, for instance, in fibres blocked with 11 mM-K Ringer's solution, or with procaine or stimulated with 500 c/s. In these cases the threshold field strength for the appearance of the wave-like contractions was, however, somewhat higher.

The effectiveness of the transverse a.c. field

Katz & Lou (1947) have shown that a transversely applied a.c. field caused contraction in a muscle which had been rendered non-propagating by excess potassium. With an a.c. field of 2–4 V/cm (1000 c/s), muscles produced about 80 % of the tetanus tension, when blocked with 12 mM-K Ringer's solution (Sten-Knudsen, 1954; Csapo & Wilkie, 1956) and it was concluded that in these experiments the muscle is activated electrically without the intervention of a longitudinal current (Sten-Knudsen, 1954). Csapo & Suzuki (1958) have now suggested that these muscles were in

some kind of 'transient' state between propagating and non-propagating conditions. If so, the experimental fact that a high-frequency transverse field produces contraction should be evaluated with caution. This suggestion is said to be substantiated by their claim that the high-frequency transverse field only causes contraction in a narrow range of K concentrations equal to or just above the minimum blocking concentration. And it is stated that a further depolarization of the membrane by only a few millivolts, e.g. by addition of 1–2 mM excess K to the Ringer's solution, abolished altogether the stimulating effect of the high-frequency transverse field.



Text-fig. 3. Superimposed tracings of tension–field strength curves with m.ext. long.dig. IV soaked in excess K Ringer's solution, the numbers on the curves indicating the K concentrations (mM). Ordinate, isometric tension (g); abscissa, transverse a.c. field strength (V/cm, 600 c/s). Dotted line, maximum isometric tension. For further explanation see text.

It is difficult to imagine how a transverse a.c. field could restore impulse conduction in a muscle blocked with 12 mM-K Ringer's solution and in fact no action potentials are observed experimentally (Sten-Knudsen, unpublished). It was therefore considered desirable to see if Csapo & Suzuki's observations could be confirmed. In experiments of this type it is important that a reduced mechanical response, caused by excessive stimulation, should not be confused with effects of excess K concentrations on the force of contractions. Therefore, to reduce the total duration of stimulation at each K concentration, the muscle was stimulated for 2 sec, with the a.c. field continuously increasing from zero to maximum field strength. Direct recording of force as a function of field strength was made by connecting the a.c. voltage across the electrodes (two-way rectified and filtered)

to the X-plates of the oscilloscope, while tension was recorded on the Y-plates. In Text-fig. 3 are shown superimposed tracings of tension–field strength curves (at 600 c/s) with the m.ext.long.dig. IV soaked in excess K Ringer's solution, the numbers on the curves indicating the K concentrations in millimoles. Before each a.c. stimulation the muscle was soaked in the excess K Ringer's solution for more than 15 min to let it equilibrate, and before changing to a new K concentration the muscle was soaked in normal Ringer's solution for about half an hour and then checked for the tetanus tension, indicated by the dotted line in Text-fig. 3. The effect of the excess K Ringer's solution consists partly in shifting the curves to the right, along the field axis, and partly in reducing the maximum tension. Thus, with 11 mM-K (membrane potential, according to Adrian (1956), −62 mV), threshold was about 1 V/cm and maximum tension 93 % of the normal tetanus tension, while with 18 mM-K (membrane potential: −50 mV) threshold was 2·5 V/cm and maximum tension 53 % of the tetanus tension. Although the mechanical response of the non-propagating muscle decreased with increasing K concentrations, the muscle was nevertheless able to develop an appreciable amount of tension, even when soaked in 18 mM-K Ringer's solution. Hence, the present results do not confirm the observations of Csapo & Suzuki (1958), according to which high-frequency transverse stimulation was only effective at K concentrations equal to or less than 12 mM.

DISCUSSION

Recently Csapo (1957) and Csapo & Suzuki (1958) have attempted to revive the now obsolete 'window field' theory of Bay *et al.* (1953) by putting forward the hypothesis that it is the *combined action* of depolarization and internal current flow which causes contraction in a muscle fibre. The modified 'window field' theory is said to be consistent with the view that the fibrils within the fibre are stimulated to contract by the longitudinal current flowing through them, e.g. during the passage of an action potential along the fibre. In this hypothesis depolarization is considered necessary, but not sufficient; in addition, internal current flow (assumed to be ineffective with normal membrane potential) is required to cause the fibrils to contract. This concept has also been advocated at some length in a recent review (Gelfan, 1958). What Csapo & Suzuki (1958) appear to have done is to modify the 'window field' theory of Bay *et al.* (1953) so as to make it consistent with some of the evidence which was brought forward against it in its original form. However, their supporting evidence differs in essential aspects from the results of all the other investigations in the same field (Katz & Lou, 1947; Sten-Knudsen, 1954; Csapo & Wilkie, 1956; Hodgkin & Horowicz, 1956). The purpose of the present study is twofold, (i) to test whether Csapo & Suzuki's theory is compatible with

the available evidence and (ii) to find out whether Csapo & Suzuki's own results require a new theory or can readily be explained on the more commonly held view, namely, that sudden depolarization of the surface membrane of the fibre is responsible for eliciting contraction (see, for example, Katz, 1950).

Csapo & Suzuki assign to the longitudinal field of the action potential the role of moving ions over a distance of 2·5 μ, i.e. over the length of a sarcomere, and claim that the maximum gradient of the action potential (about 3 V/cm) can move Ca^{2+} over a distance of 2 μ within a period of 30 msec. Now suppose that the mobilities of Ca^{2+} and K^+ within the cell are of the same order of magnitude, which for K^+ is about 5×10^{-4} cm.sec^{-1}/V.cm^{-1} (Hodgkin & Keynes, 1953). A field of 3 V/cm will then move the ions over a distance of only 0·45 μ in 30 msec ($= 5 \times 10^{-4} \times 3 \times 3 \times 10^{-2}$ cm). But Csapo & Suzuki have ignored the fact that this maximum field of 3 V/cm is not maintained for 30 msec, but only for about 0·3 msec, after which it is immediately reversed in sign. Thus, the maximum displacement of a Ca^{2+} ion during the passage of an action potential is likely to be not 2 μ but of the order of 0·0045 μ. This does not rule out the possibility that some internal ionic displacement may be instrumental in activating the contractile material, though it cannot be anything like the value stated.

The original 'window field' theory proposed by Bay *et al.* (1953) was opposed on the grounds that, in the absence of propagation, internal current flow causes contraction only at points on the fibre where the membrane is being depolarized and not along its whole length (Kuffler, 1946; Sten-Knudsen, 1954; Huxley & Taylor, 1955, 1958; Watanabe & Ayabe, 1956). The modified 'window field' theory of Csapo & Suzuki (1958) is consistent with this set of observations, although it is difficult to reconcile this theory with the observation that a longitudinal d.c. field, when applied to a muscle in moderate contracture, causes relaxation in part of the anodic region, while contraction is enhanced only in the cathodic region (Kuffler, 1946; Sten-Knudsen, 1954). But, to make Csapo & Suzuki's theory applicable in general, it would follow that membrane depolarization never can cause contraction in a muscle fibre without the intervention of a strong internal current flow. Now, it is possible to depolarize a muscle fibre along its whole length and at the same time to prevent any internal current flow parallel to the longitudinal axis of the myofibrils. Nevertheless, in such cases full, or almost full, tetanus tension is obtained. This has been accomplished in two ways:

(1) When single fibres are made non-conducting by Na-free Ringer's solution, a rapid application of high K solution gives maximum tension (Hodgkin & Horowicz, 1956). Csapo & Suzuki (1958) reject this experi-

ment as inconclusive by suggesting that effective internal currents were generated during the longitudinal application of the K-rich solution. It is clearly open to them to repeat the Hodgkin–Horowicz experiment with transverse application of the high-K Ringer's solution.

(2) Nearly the full tetanus tension can be elicited in a non-propagating muscle by stimulating it transversely with an a.c. field (Katz & Lou, 1947; Sten-Knudsen, 1954; Csapo & Wilkie, 1956). In this case there is no longitudinal internal current, and the transverse current can also be made quite small. Csapo & Suzuki (1958) reject this evidence and suggest that fibres blocked with 12 mM-K Ringer's solution, are in a 'transient' state between propagating and non-propagating condition. Further they assert that an addition of 1–2 mM-K, i.e. a further depolarization by only a few millivolts, abolishes altogether the effectiveness of the transverse field. The results of the present investigation show that this statement is incorrect, since a muscle soaked in 18 mM-K Ringer's solution produced an appreciable amount of tension (50 % of tetanus tension) when stimulated with a transverse field (600 c/s). It is not possible to see how Csapo & Suzuki obtained their contrary results, for they did not state the conditions under which their observations were made. Perhaps they failed to recognize that increasing the K concentration shifts the tension–field-strength curves, as is shown in Text-fig. 3. Thus, with a field strength of 3 V/cm, tension was 87 % of the tetanus tension when the muscle was soaked in 11 mM-K Ringer's solution; in 18 mM-K Ringer's solution the response amounted only to 7 %, but a maximum tension of 50 % tetanus tension could still be developed at this concentration if adequate field strengths were used.

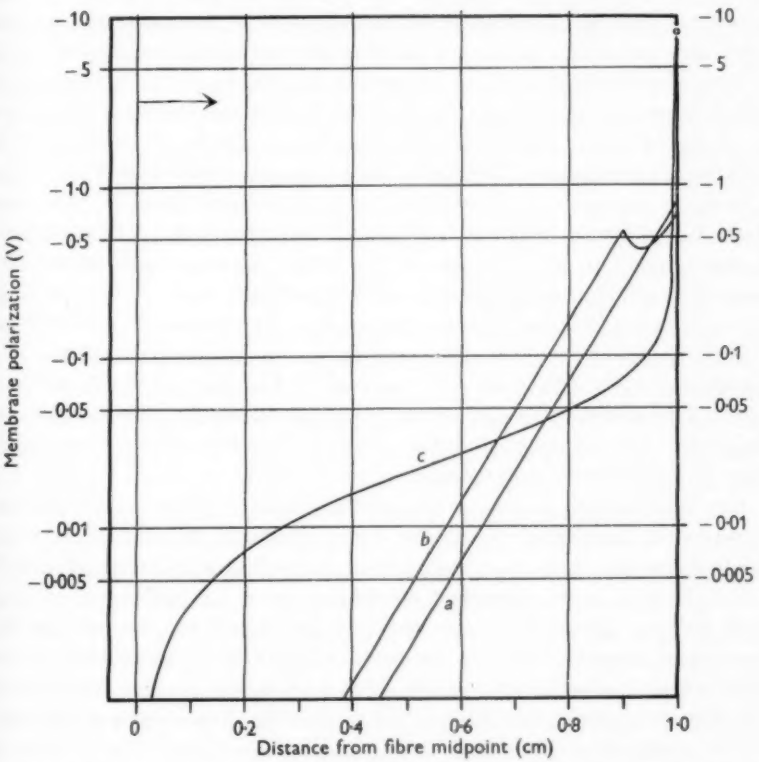
Csapo & Suzuki (1958) have been unable to invalidate the observations mentioned in (1) and (2), which are clearly at variance with their theory. That a non-propagating muscle contracts at the cathodic region in a longitudinal electric field has been observed by various investigators during the course of the past hundred years (Wundt, 1859; Kühne, 1860; du Bois-Reymond, 1875; Jendrássik, 1879; Hermann, 1886; Kuffler, 1946; Sten-Knudsen, 1954), Csapo & Suzuki (1958) representing the sole exception. Thus, except for their own results, Csapo & Suzuki's modified theory remains inconsistent with all the evidence which is at present available.

A result which Csapo & Suzuki cite in favour of their theory is the observation that only a small fraction of the tetanus tension was produced by soaking a whole muscle in excess-K Ringer's solution. But this fact has been well known to other workers and it has usually been ascribed to the fact that it takes time for potassium to diffuse into a muscle, during which the contractile response declines (see e.g. Hodgkin & Horowicz, 1956). Csapo & Suzuki's main evidence, obtained on a whole sartorius

muscle can be summarized as follows: (1) Longitudinal a.c. stimulation was only effective if the non-propagating muscle was deeply depolarized by the excess-K Ringer's solution. (2) At threshold field strength (4 V/cm, 60 c/s) only the middle portion shortened. (3) With strong a.c. fields (6–20 V/cm) the middle portions shortened more than the rest. And (4) during propagated activity shortening was strongest in the middle portion (some 30 %). The experimental conditions of the experiments reported in this paper were quite similar to those of Csapo & Suzuki (1958), except that single fibres were used. This makes it possible to align the fibre in the direction of the field and provides a better discrimination of changes in different regions along the fibre. The experiments showed that *contraction was always strongest at the end regions* and, with field strengths in the range of 0·7–5·5 V/cm (100 c/s), localized solely to these parts of the fibre. The results are clearly at variance with the Csapo–Suzuki theory. However, it was also found in the present work that large fields (e.g. higher than 5·5 V/cm, 100 c/s) caused the fibre to shorten along its whole length. This is the only observation which resembles those of Csapo & Suzuki, but the contraction is only slightly similar to a normal tetanus and its origin can be adequately explained in terms of changes at the membrane. It thus remains an open question whether this observation alone necessitates the introduction of a radically different theory of activation.

When a parallel-fibred muscle is placed longitudinally in a homogeneous d.c. field, simple application of the core conductor equations (Hodgkin & Rushton, 1946) shows that almost the total current which flows in the fibre must enter and leave at its terminal region. These results were used to describe qualitatively the way a non-propagating muscle was activated when placed longitudinally in a homogeneous d.c. field (Sten-Knudsen, 1954). Csapo & Suzuki (1958) assumed the same results for the somewhat different situation where the field was alternating. The solution of the core conductor equations for this case (see Appendix 1) shows that the amplitude of the voltage oscillations across the membrane diminishes towards the fibre mid point according to the same general law as in the d.c. case, with the exception that (*a*) the parameter for the attenuation (the a.c. space constant) now is the square root of twice the ratio between the membrane a.c. resistance of one unit length and the longitudinal internal resistance per unit length, (*b*) there is a progressive phase shift of the voltage fluctuations along the length of the fibre. In Text-fig. 4 (curve *a*) is shown the amplitude–distance relationship of the membrane polarization (disregarding phase relations) during the peak of a longitudinal a.c. field (100 c/s), computed from the numerical data of Katz (1948) and Nicholls (1956). During each half cycle, activation will occur only in the region which is sufficiently depolarized, causing a contraction which will

last as long as a normal twitch. This contraction, once started, will not be altered by the following hyperpolarizing phase. In agreement with the findings in the present investigation it is therefore to be expected that the fibre will shorten only at its ends as long as the field strengths are moderate. If the fibre is depolarized close to its contracture threshold with excess-K Ringer's solution, only a small additional depolarization, say 5–10 mV, is needed to cause activation. It was to a muscle in this condition that Csapo & Suzuki (1958) applied strong fields (6–20 V/cm), the implications of which were apparently not fully realized by them. It appears from curve *a* in Text-fig. 4 that a field of 10 V/cm is likely to produce activation in



Text-fig. 4. Calculated amplitude–distance relationship of membrane polarization on a fibre 2 cm long during the peak of a longitudinal a.c. field (10 V/cm, r.m.s., 100 c/s). Ordinate, membrane polarization, log scale; abscissa, distance from fibre mid point. *a*, application of the core-conductor equations assuming constant parameters along the fibre length; *b*, the effect of a reduced space constant in the terminal 1 mm portion of the fibre; *c*, the fibre tapering in the shape of a long prolate ellipsoid. For further explanation, see text and appendix.

about one-fifth of the fibre at each end, but none in the middle. As was mentioned earlier, any observed shortening in the middle can be adequately accounted for by such complicating geometrical factors as tapering of the fibre towards its ends, sudden irregularities in fibre diameter and misalignment of the fibre in the direction of the field. It is possible that Csapo & Suzuki (1958) were led to their conclusions because they assumed (without explicitly stating so) that these second-order effects could be neglected; but they can, in fact, be shown to be far from negligible. In the first place, tapering of the fibre towards its ends results both in an increased membrane polarization at the end regions and in a less steep decline of polarization towards the mid point of the fibre. This is illustrated in curve *b*, Text-fig. 4, which shows the effect of a reduced space constant in the terminal 1 mm portion of the fibre corresponding, e.g. to a reduction in fibre diameter by half (see Appendix 2), and in curve *c*, Text-fig. 4, which shows the membrane polarization in a fibre gradually tapering in the shape of a long prolate ellipsoid (see Appendix 4). In the latter case the area of membrane polarization has increased so that a field of 10 V/cm is likely to activate about 80 % of the whole muscle fibre. In the second place it is scarcely possible to place all the fibres of a whole sartorius muscle parallel to the direction of the field. With an angle of misalignment of 5°, which is easily possible with this muscle, and a field of 10 V/cm, the transverse component of the field is $E_t = 14{\cdot}14 \times \sin 5° = 1{\cdot}233$ V/cm. This component of the field will cause an a.c. membrane polarization along the whole length of the fibre, the peak value of which is 12 mV (see Appendix 3). This additional depolarization will be sufficient to cause activation also in the mid region of the fibre, where a properly aligned fibre will not show any membrane polarization.

The effect which a strong longitudinal field (10 V/cm, or more) produces on the membrane, especially at the fibre ends, was also neglected by Csapo & Suzuki. A simple consideration shows that with a field of 10 V/cm, r.m.s. (100 c/s) and a muscle 3 cm long a potential difference of about 10 V will be produced across the extreme terminal part of the fibre membrane, provided that its dielectric strength is not exceeded. In view of this fact it was hardly surprising to find in the present investigation that the fibre gradually deteriorated at its ends. The irregular shortening which occurred at high field strengths displayed a similarity with the 'galvanic waves' of the type described by Hermann (1886). They are probably due to the local increase in thickness of the extremely shortened fibre ends, causing more current to enter this region as a result of its decreased longitudinal internal resistance. Under certain conditions, depending upon the degree of change in the electric constants in the region of bulge, part of this current will be forced to leave the fibre again in the

region next to the bulge and thus to create a new cathodic region which causes the local contraction to move along the fibre (see Appendix 2).

Thus it is most likely that Csapo & Suzuki's (1958) findings are due to the several complicating factors in their experimental situation. The sartorius muscle was depolarized close to its contracture threshold by the 16 mM-K Ringer's solution. The combination of great field strengths, misalignment of the fibres in the direction of the field, which inevitably occurs with a sartorius muscle, and irregularities of the fibre diameters which are unavoidable at their ends, provided sufficient depolarization along the whole length of the fibre to produce a contraction at all points of the fibre. It may be that part of the response which Csapo & Suzuki (1958) observed was of the 'galvanic wave' type, since the irregularity of the movements of the individual fibres would pass unnoticed. In their sartorius muscle of 3 cm length they observed a threshold of 4 V/cm for an over-all contraction. To produce the same potential drop along a fibre 2 cm long (as in the present case) the longitudinal field must be raised to about $4 \times 3/2 = 6$ V/cm. This is close to the threshold for the 'galvanic wave' contraction in the present experiments, while the threshold for the contraction at the fibre end was only 0·73 V/cm (equivalent to 0·5 V/cm in the 3 cm long sartorius muscle). Furthermore, excessive potential differences which must have existed across the membrane at the end-regions of the fibre in Csapo & Suzuki's experiments (1958) may partly explain their finding that contraction was strongest in the middle of the muscle. Even if the ends were not responding optimally it would still be possible to achieve adequate activation of the rest of the fibre. Therefore, with proper regard to the various complexities in Csapo & Suzuki's experimental conditions, their results cannot be considered to contradict, but rather to substantiate, the view that it is membrane depolarization, regardless of internal longitudinal current flow, which sets off contraction. And their results cannot be said to necessitate a new theory of activation.

Szent-Györgyi (1956) considers the stimulating effect of transverse a.c. stimulation to be due to some internal disturbance of electron distribution of the contractile proteins caused by the transverse current, rather than to the effect of successive depolarizing half cycles. The Watanabe–Ayabe (1956) experiment (current flow between two micro-electrodes in the interior of the fibre gives no contraction) casts doubt on this concept, because in this experiment there must have been a considerable transverse component of the current around each electrode. Pointing in the same direction is the observation that the threshold of transverse stimulation increases with the frequency; a rough calculation shows that the internal transverse field in the range of frequencies used must have increased by almost 170 times (C. Guld & O. Sten-Knudsen, unpublished)

There is strong evidence (Huxley & Taylor, 1955, 1958) that the disturbance of the surface membrane is conducted inwards along some structures in the middle of the I bands. Huxley (1957) has tentatively suggested that (*a*) the mechanism of conduction is an electrical one taking place in a cable-like structure and (*b*) activation is in some way conveyed from the channels to the action filaments as a result of a change in the potential difference across the walls. According to this scheme the internal current flow which elicits contraction is that which traverses the walls of the channels, possibly by moving some particular ion from one compartment to the other. If this scheme is correct it also explains why Szent-Györgyi's 'window field' has been found ineffective in causing contraction. It is natural to assume that the threshold for the change in potential difference across the channel walls is equal to that across the whole fibre membrane, say 10 mV in a K-blocked fibre. To produce this amount of depolarization on a channel wall of 100 Å diameter requires a 'window-field' strength which is, approximately at least, $E = 0{\cdot}5 \times 10^{-2}/10^{-6} = 5000$ V/cm (see Appendix 3, eqn. 3.11), which is far above anything which can be obtained experimentally without damaging the fibre.

SUMMARY

1. Csapo & Suzuki have put forward the theory that it is the combined action of depolarization *and* internal current flow which elicits contraction. This is a modification of the now discarded 'window field' theory of Bay *et al.* (1953). Membrane depolarization is considered a necessary 'priming' step which by itself is ineffective in setting off contraction. This theory is said to be consistent with the experimental facts. But to support the theory Csapo & Suzuki have presented observations which differ in essential aspects from those of other investigators.

2. The experiments of Csapo & Suzuki have been repeated on isolated single fibres, by means of a photographic technique which provided good resolution of events taking place at different points along the length of the fibre. Csapo & Suzuki's main observation could not be reproduced, namely that a longitudinal a.c. field, when applied to a muscle placed in a high-K Ringer's solution, causes the greatest amount of shortening in the middle portion. Neither could their claim be confirmed that transverse a.c. stimulation is ineffective if the external K concentration is higher than 12 mM.

3. Csapo & Suzuki's theory is shown to be inconsistent with the evidence at present available. It is further shown that their results do not require a new theory of activation, but can be explained on the current view, that it is membrane depolarization which initiates contraction.

4. In a theoretical Appendix is derived the form of the core-conductor

equation appropriate for alternating fields. It is also shown how the distribution of membrane polarization varies according to the shape of the fibre.

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APPENDIX

Calculation of the membrane polarization of a muscle fibre placed in a homogeneous a.c. field

Let

E = $\mathscr{E}\cos\omega t$ = undisturbed parallel a.c. field (V/cm); $\omega = 2\pi\nu$, where ν is the frequency (c/s);

x, y, z = Cartesian reference system placed with the z-axis coinciding with the direction of the fibre axis and the origin being placed at the mid point of the fibre;

r, ϕ, z = cylindrical co-ordinate reference system defined by: $x = r\cos\phi$, $y = r\sin\phi$, $z = z$, where ϕ is the angle between the x-axis and r.

η, θ, ϕ = prolate spheroidal co-ordinate reference system (cf. Hobson, 1931) defined by: $z = f\cosh\eta\cos\theta$, $x = f\sinh\eta\sin\theta\cos\phi$, $y = f\sinh\eta\sin\theta\sin\phi$, where f is the focal distance. η is the parameter for a family of confocal prolate ellipsoids: $(z/f\cosh\eta)^2+(x^2+y^2)/(f\sinh\eta)^2 = 1$, $1 \leqslant \eta < \infty$, and θ is the parameter for the orthogonal system of confocal hyperboloids:

$$(z/f\cos\theta)^2-(x^2+y^2)/(f\sin\theta)^2 = 1, \quad 0 \leqslant \theta \leqslant \pi.$$

A point in space is specified as the intersection between these surfaces with a plane passing through the z-axis and making an angle ϕ with the x-axis;

$2L$ = length of the fibre (cm);

a = radius of the fibre (cm);

ψ_i = potential of myoplasm caused by E (V);

ψ_e = potential in Ringer's fluid (V);

V_m = potential difference across the membrane in excess of the normal resting membrane potential = $\psi_e(a)-\psi_i(a)$, (V);

I = longitudinal current in myoplasm, positive in the direction of increasing z (A);

I_m = current entering the myoplasm per unit length (A/cm);

R_i = longitudinal resistance of myoplasm per unit length (Ω/cm);

R_m = membrane resistance of one unit length (Ω.cm);

C_m = membrane capacitance per unit length (F/cm);

ρ_i = specific resistivity of myoplasm (Ω.cm);

ρ_m = specific surface resistivity of membrane ($\Omega.\text{cm}^2$);

c_m = capacitance per unit area of membrane (F/cm^2);

ρ_e = specific resistivity of Ringer's fluid = 100 Ω.cm at 20° C;

t = time (sec);

i = $\sqrt{-1}$.

The following average values are used (Katz, 1948; Nicholls, 1956):

$$\rho_m = 4\,\text{k}\Omega.\text{cm}^2, \quad \rho_i = 300\,\Omega.\text{cm}, \quad c_m = 4\,\mu\text{F/cm}^2.$$

1. *Application of the core conductor equations to calculate membrane polarization caused by a longitudinal a.c. field, assuming constant parameters*

We have from the fundamental core conductor equations (Hodgkin & Rushton, 1946)

$$I_m = \frac{V_m}{R_m}+C_m\frac{\partial V_m}{\partial t} = \frac{\partial I}{\partial z} = -\frac{1}{R_i}\frac{\partial^2\psi_i}{\partial z^2},$$

Since the diameter of the fibre is small as compared to its length, the disturbing effect of the fibre upon the parallel field is small. As a first order of approximation we have therefore: $\psi_e(z, a) = -Ez$ and $\partial^2\psi_i/\partial z^2 = \partial^2(-Ez - V_m)/\partial z^2 = -\partial^2 V_m/\partial z^2$. The equation to be solved for V_m is therefore

$$\frac{\partial^2 V_m}{\partial z^2} = R_i C_m \frac{\partial V_m}{\partial t} + \frac{R_i}{R_m} V_m, \tag{1.1}$$

together with the conditions: (i) $V_m(0) = 0$, (ii) $I(L) = I(-L) = 0$, or $\partial V_m/\partial z = -E$, for $z = \pm L$, and (iii) $E = \mathscr{E}\cos\omega t$. Condition (ii) is equivalent to the assumption that no current enters at the end surfaces of the fibre. To find the stationary solution we seek, as originally suggested by Stokes (1856), a solution of eqn. (1.1) of type

$$V_m = Z(z)\exp(i\omega t), \tag{1.2}$$

where $Z(z)$ is a function of z alone. Substituting eqn. (1.2) into eqn. (1.1) it follows that Z must satisfy

$$\frac{\mathrm{d}^2 Z}{\mathrm{d}z^2} - \frac{Z}{\lambda_c^2} = 0, \tag{1.3}$$

where λ_c is the complex space constant

$$\lambda_c^{-1} = \sqrt{\{\omega R_i C_m (i + 1/\omega C_m R_m)\}}. \tag{1.4}$$

At 100 c/s, $\omega C_m R_m = \omega c_m \rho_m = 10$, hence

$$\lambda_c^{-1} \approx \sqrt{\{i\omega C_m R_i\}} = (1+i)\sqrt{\{\omega R_i C_m/2\}} = (1+i)m.$$

The solution of eqn. (1.3) which satisfies (i), (ii) and (iii) is

$$V_m = -\mathscr{E}\frac{\sinh[z(1+i)m]}{m(1+i)\cosh[L(1+i)m]}\exp(i\omega t). \tag{1.5}$$

The real part of eqn. (1.5) may be reduced to the form

$$V_m = -\frac{1}{\sqrt{2}\,m}\mathscr{E}\sqrt{\left\{\frac{\sinh^2(mz)+\sin^2(mz)}{\cosh^2(mL)-\sin^2(mL)}\right\}}\cos(\omega t+\gamma(z)-\gamma_1-\pi/4), \tag{1.6}$$

where $\tan\gamma(z) = \tan(mz)\coth(mz)$ and $\tan\gamma_1 = \tan(mL)\tanh(mL)$. At 100 c/s, $m = \sqrt{(\omega C_m R_i/2)} = 12{\cdot}2$. For $L = 1$, $\cosh(mL) \sim 10^5 \gg \sin(mL)$ and $\sinh^2(mz) \gg \sin^2(m)$ for $z > 2$ mm. Eqn. (1.6) is therefore equivalent to

$$V_m = -\frac{1}{\sqrt{2}}\mathscr{E}\frac{\sinh(mz)}{m\cosh(mL)}\cos(\omega t+\gamma(z)-\gamma_1-\pi/4), \tag{1.7}$$

which shows that the amplitude of the oscillations of V_m diminishes like $\sinh(mz)$, which in principle is equivalent to the steady d.c. case with the exception that the space constant which governs the attenuation is $1/m = \sqrt{(2/\omega C_m R_i)}$ instead of $\lambda = \sqrt{(R_m/R_i)}$. On the other hand there is a progressive shift in the phase of V_m along the length of the fibre. However, if only maximal values of the voltage fluctuations across the membrane are considered they can satisfactorily be described by the equation of the well-known form

$$\frac{\mathrm{d}^2 V_m}{\mathrm{d}z^2} - m^2 V_m = 0, \tag{1.8}$$

where m^{-2} is twice the ratio between the membrane a.c. resistance and the longitudinal internal resistance per unit length.

2. *The effect of irregularities in the fibre diameter*

Since $R_i = \rho_i/\pi a^2$, $R_m = \rho_m/2\pi a$, $C_m = c_m 2\pi a$, any local change in a will change the pattern of current entry into the fibre. Let it be assumed, for the sake of simplicity, that the irregularity is symmetric with respect to the mid point of the fibre such that in the region $|l| \leqslant |z| \leqslant |L|$ we have $V_m = F(z)$, $R_i = R_0$, $m = m_0$; while in the region $-l \leqslant z \leqslant l$ we have $V_m = G(z)$, $R_i = R_1$, $m = m_1$.

The proper solution of eqn. (1.8) for F is

$$F(z) = A \exp(m_0 z) + B \exp(-m_0 z), \tag{2.1}$$

while that for G is

$$G(z) = C \sinh(m_1 z), \tag{2.2}$$

where A, B and C are constants to be determined from the boundary conditions: (i) $\mathrm{d}F/\mathrm{d}z = -E$, for $z = L$; (ii) $F(l) = G(l)$, and (iii) $(E + \mathrm{d}F/\mathrm{d}z)/R_0 = (E + \mathrm{d}G/\mathrm{d}z)/R_1$ for $z = l$. (ii) and (iii) are equivalent to the conditions that both V_m and I must be continuous at $z = l$. Insertion of eqns. (2.1) and (2.2) into (i), (ii) and (iii) gives three simultaneous equations for the determination of A, B and C from the values of which the final solution may be given as

$$F(z) = -\frac{1}{\sqrt{2}}\mathscr{E}\frac{\alpha\cosh(m_1 l)\sinh m_0(z-l) + [\cosh m_0(z-l) - \beta\cosh m_0(L-z)]\sinh(m_1 l)}{m_0[\sinh(m_1 l)\sinh m_0(L-l) + \cosh(m_1 l)\cosh m_0(L-l)]},$$

$$G(z) = -\frac{1}{\sqrt{2}}\mathscr{E}\frac{[1 - \beta\cosh m_0(L-l)]\sinh(m_1 z)}{m_0[\sinh(m_1 l)\sinh m_0(L-l) + \cosh(m_1 l)\cosh m_0(L-l)]},$$

where $\alpha = R_0 m_1/R_1 m_0$; $\beta = 1 - R_0/R_1$. If $\beta\cosh m_0(L-l) > 1$, part of the current which has entered the fibre will leave again already in the transition region near $z = l$.

3. *Transverse field*

The fibre is regarded as an infinite circular cylinder of radius a and internal conductivity $1/\rho_i = g_i$ (mho/cm) covered with an infinitely thin layer of surface resistivity ρ_m. The x-axis is placed in the direction of the undisturbed field, E, the potential of which is $\psi_0 = -Ex$. The current density has nowhere a z-component and the potential function ψ of the current field must in all space satisfy the two-dimensional Laplace equation

$$\nabla^2\psi = \frac{\partial}{\partial r}\left(r\frac{\partial\psi}{\partial r}\right) + \frac{1}{r}\frac{\partial^2\psi}{\partial\phi^2} = 0, \tag{3.1}$$

together with the conditions: (i) the disturbing effect of the cylinder on the original parallel field vanishes at great distances from the origin, so: $\psi \to -Ex = -Er\cos\phi$, for $r \to \infty$. (ii) $\psi(r, \phi') = \psi(r, -\phi)$, since ψ must be symmetrical with respect to the $x-z$ plane. (iii) ψ remains finite at the origin and throughout the whole space the $y-z$ plane is an equipotential which arbitrarily may be assigned the value zero, so $\psi = 0$ for $\phi = \pm\frac{1}{2}\pi$ and all r. From the general solution of eqn. (3.1)

$$\psi = (A_0\phi + B_0)(C_0\ln r + D_0) + \Sigma(A_n r^n + B_n r^{-n})(C_n\cos n\phi + D_n\sin n\phi), \tag{3.2}$$

(cf. Smythe, 1950). We seek solutions for ψ_e and ψ_i which satisfy conditions (i), (ii) and (iii). These are

$$\psi_e = -Er\cos\phi + \Sigma B_n r^{-n}\cos n\phi, \tag{3.3}$$

$$\psi_i = \Sigma A_n r^n\cos n\phi. \tag{3.4}$$

Since the membrane conductivity $\delta a/\rho_m$ (where δa is the membrane thickness) is much smaller than g_e and g_i, practically no current will flow tangentially within the membrane. Therefore, the normal component of the current density flowing across the interface between the outer side of the membrane and the exterior region, $-g_e(\partial\psi_e/\partial n)_a$, must be equal to that, $-g_i(\partial\psi_i/\partial n)_a$, flowing across the inner membrane into the fibre. The boundary conditions to be satisfied for ψ_e and ψ_i are thus

$$g_e\left(\frac{\partial\psi_e}{\partial r}\right)_a = g_i\left(\frac{\partial\psi_i}{\partial r}\right)_a, \tag{3.5}$$

since $\partial/\partial n = \partial/\partial r$, and

$$\psi_i(a, \phi) - \psi_e(a, \phi) = -g_i\rho_m\left(\frac{\partial\psi_i}{\partial r}\right)_a. \tag{3.6}$$

To determine the constants A_n and B_n, eqns. (3.3) and (3.4) are inserted into (3.5) and (3.6).

It can be shown that all the coefficients vanish identically to zero except those for $n = 1$, the determination of which is given by the two simultaneous equations

$$g_i A_1 + a^{-2} g_e B_1 = -g_e E, \tag{3.7}$$

$$(a + g_i) A_1 - a^{-1} B_1 = -aE. \tag{3.8}$$

Solving these equations for A_1 and B_1 and inserting into eqn. (3.3) and (3.4) gives

$$\psi_e = -Er\cos\phi - \frac{a^3}{r}\frac{1 + \rho_m g_i/a - g_i/g_e}{1 + g_i/g_e + \rho_m g_i/a} E\cos\phi, \tag{3.9}$$

$$\psi_i = -\frac{2}{1 + g_i/g_e + \rho_m g_i/a} Er\cos\phi, \tag{3.10}$$

from which it follows that the membrane polarization, $V_m = \psi_e(a, \phi) - \psi_i(a, \phi)$, is

$$V_m = -2\frac{\rho_m g_i/a}{1 + g_i/g_e + \rho_m g_i/a}(aE)\cos\phi \tag{3.11}$$

At 100 c/s, ρ_m, can be replaced solely by the a.c. surface resistivity of the membrane ($= 400\ \Omega.\mathrm{cm}^2$), and for a fibre of 100 μ in diameter eqn. (3.11) becomes

$$V_m = -0{\cdot}99 \times 10^{-3} E\cos\phi. \tag{3.12}$$

4. *The effect of tapering along the fibre*

To solve this case by means of the core conductor equations, R_i, R_m and C_m must be considered functions of the distance z along the fibre. The fundamental equation takes then the modified form

$$\frac{\partial^2 V_m}{\partial z^2} - \frac{1}{R_i}\frac{\mathrm{d}R_i}{\mathrm{d}z}\frac{\partial V_m}{\partial z} = \frac{R_i}{R_m} V_m + R_i C_m \frac{\partial V_m}{\partial t} + \frac{1}{R_i}\frac{\mathrm{d}R_i}{\mathrm{d}z} E, \tag{4.1}$$

However, even in the case of a steady d.c. field, eqn. (4.1) can only be solved in terms of known functions by assuming a special regular geometry of the fibre. In the simplest case where the fibre tapers linearly towards the ends at an angle α, the general solution of eqn. (4.1) takes the form (the independent variable z has been replaced by the fibre radius r)

$$V_m(r) = A\mathrm{I}_1(k\sqrt{r})/\sqrt{r} + B\mathrm{K}_1(k\sqrt{r})/\sqrt{r},$$

where $k^2 = 4R_m/R_i\alpha^2$ and I_1 and K_1 are the modified Bessel functions of the first order, but this expression is not very suited for practical computations.

If the fibre is considered as a long prolate ellipsoid it is possible to obtain a first-order approximation of the membrane polarization by a straightforward solution of the volume conduction equations as in 3. A short outline of the procedure is as follows: The potential ψ is calculated in prolate spheroidal co-ordinates. Since ψ is independent of ϕ (rotational symmetry with respect to the fibre axis) the Laplace equation takes the form

$$\nabla^2\psi = \frac{\partial^2\psi}{\partial\eta^2} + \coth\eta\frac{\partial\psi}{\partial\eta} + \frac{\partial^2\psi}{\partial\theta^2} + \tan\theta\frac{\partial\psi}{\partial\theta} = 0. \tag{4.2}$$

A solution of eqn. (4.2) of the form $\psi = V_1(\eta)\, V_2(\theta)$ makes 4.2 separate into two ordinary differential equations of Legendre's type. The proper solutions for a conducting homogeneous ellipsoid are selected and the same type of boundary conditions as (3.5) and (3.6) are applied. Since most of the current enters at the poles, the boundary conditions are adjusted to fit the present physical model correctly at the fibre axis. From these conditions ψ_e can be determined. Since the membrane polarization is $V_m = -i_m\rho_m$, where i_m is the norma component of the membrane current density, we have

$$V_m = \rho_m g_e \frac{\partial\psi_e}{\partial n} = g_e\rho_m \frac{\partial\psi_e}{\partial\eta}\frac{\partial\eta}{\partial n} \quad \text{for} \quad \eta = \eta_0, \tag{4·3}$$

where $\eta_0 = \operatorname{artanh}(a/L)$ is the ellipsoidal co-ordinate defining the fibre surface and n is

the distance measured along the normal to the ellipsoidal surface. This leads to the final result

$$-V_m = \frac{\rho_m g_i [1 - Q_1(\cosh\eta_0)/\cosh\eta_0 Q_1'(\cosh\eta_0)]}{1 + \rho_m g_i/f\cosh\eta_0 - g_i Q_1(\cosh\eta_0)/g_e \cosh\eta_0 Q_1'(\cosh\eta_0)} \left(\frac{\cosh^2\eta_0 - 1}{\cosh^2\eta_0 - \cos^2\theta}\right)^{\frac{1}{2}} E\cos\theta,$$

where Q_1 is the Legendre function of the second kind and $\cos\theta = z/L$. With $a/L = 10^{-2}$, $\cosh\eta_0$ is very nearly equal to unity, which makes $Q_1(\cosh\eta_0)/Q_1'(\cosh\eta_0) \ll 1$. Disregarding this term in the above equation and substituting $f\cosh\eta_0 = L$ it simplifies to

$$-V_m = \frac{\rho_m g_i/L}{1 + \rho_m g_i/L} \left(\frac{\cosh^2\eta_0 - 1}{\cosh^2\eta_0 - \cos^2\theta}\right)^{\frac{1}{2}} \cos\theta\,(LE). \tag{4.4}$$

For a fibre 2 cm long and 100 μ in maximum diameter $\eta_0 = \operatorname{artanh}(0{\cdot}005) = 0{\cdot}005$ and $\cosh^2\eta_0 = 1{\cdot}00002$. Using the average values of g_i and c_m and calculating ρ_m at 100 c/s ($= 400\ \Omega.\mathrm{cm}^2$) the maximum polarization which occurs at the poles is

$$V_m^0 = 0{\cdot}57 \times EL. \tag{4.5}$$

For all points on the surface except the most terminal portions ($z \leqslant 0{\cdot}9996L$) eqn. (4.4) is given with an error less than 1% by

$$V_m = -2{\cdot}55 \times 10^{-3} \cot\theta\,.EL, \tag{4.6}$$

where $\theta = \arccos(z/L)$.

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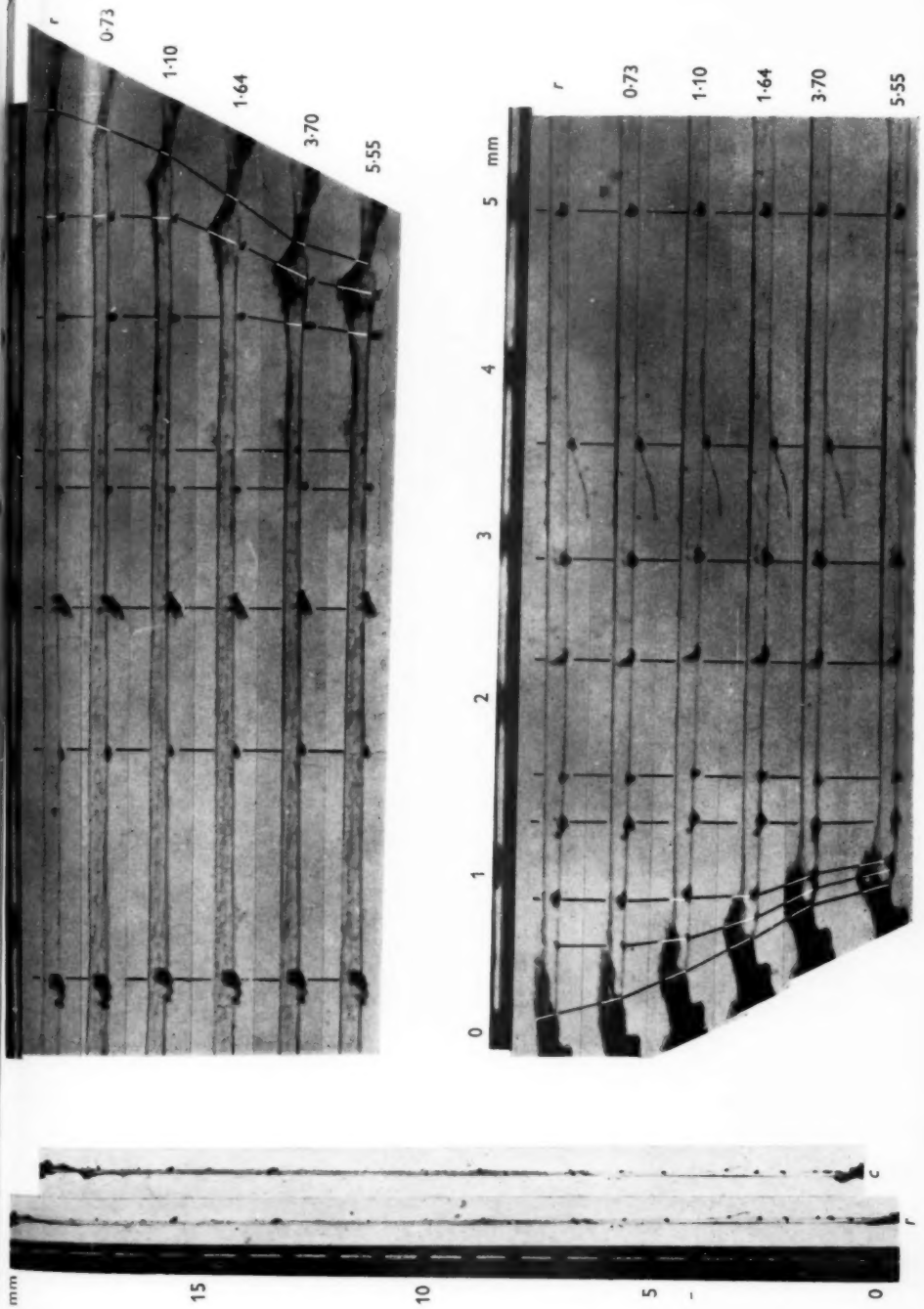
EXPLANATION OF PLATES

PLATE 1

Photographs of reversible isotonic a.c. contractures in a non-propagating isolated single fibre from the semitendinosus muscle from frog. Left-hand side: photographs of the whole length of the fibre marked with graphite particles. (*r*), resting fibre; (*c*) longitudinal a.c. field at about 5 V/cm (100 c/s). Right-hand side: enlargements of the end portions of the fibre with all the frames aligned with respect to one mark placed at a distance of about 5 mm from the ends. Upper row of each set shows the resting fibre (*r*), and those below the effect of the a.c. field, the numbers indicating the value of each field strength. The displacement of the graphite granules is shown by the black lines. For further description, see text.

PLATE 2

To show the irreversible effect of too high field strength. For explanation, see text and legend to Pl. 1. Note the difference in thickness and degree of shortening along the length of the fibre, which characterizes the 'wave-like' contractions. *b* and *b'* show the fibre ends 4 sec after stimulation with 7·3 V/cm as compared with the initial fibre length *a* and *a'*.



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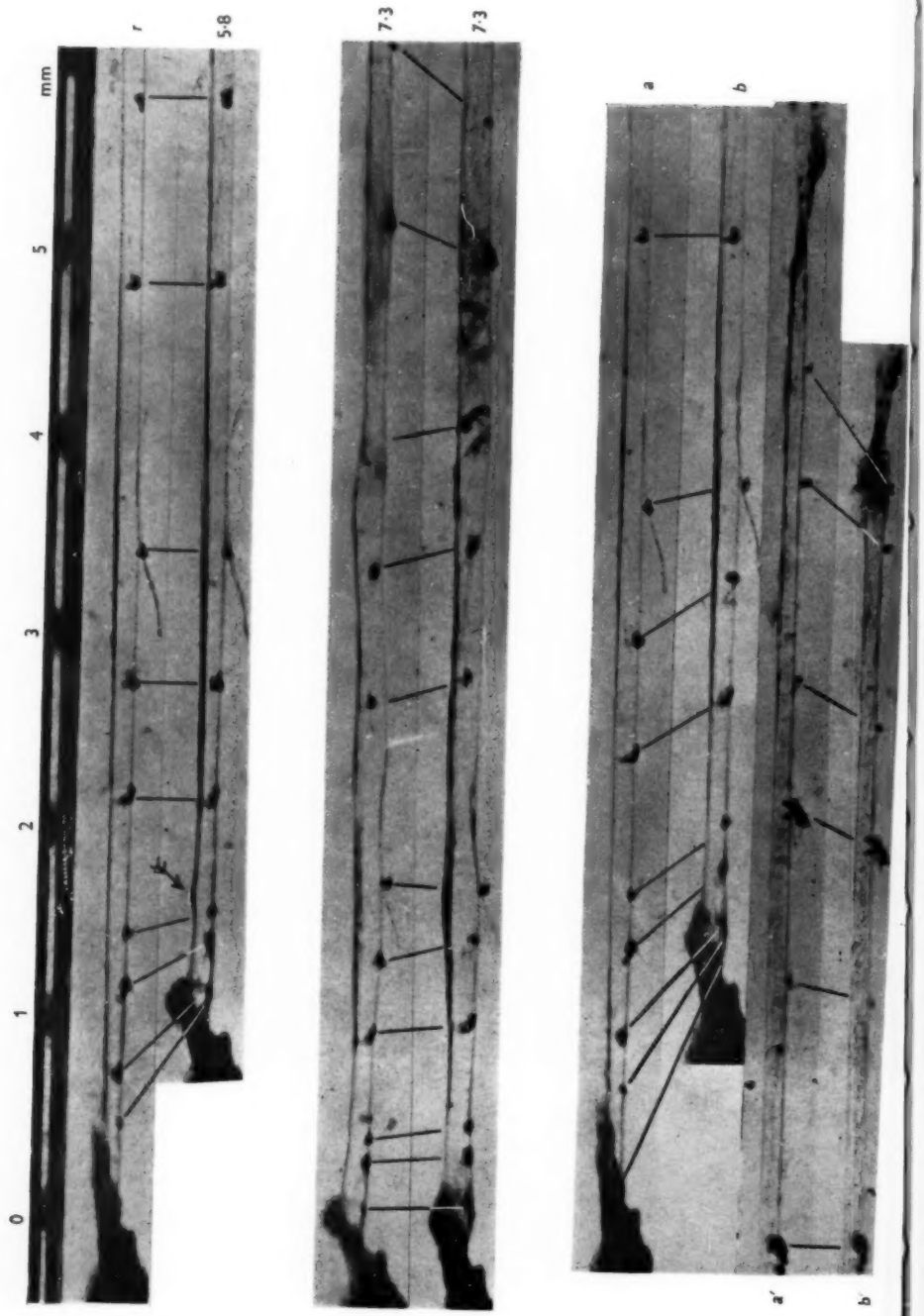

O. STEN-KNUDSEN

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EFFECTS OF PREGNANCY ON INULIN AND PARA-AMINOHIPPURATE CLEARANCES IN THE ANAESTHETIZED RAT

By B. F. MATTHEWS and D. W. TAYLOR
From the Department of Physiology, University of Aberdeen

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Earlier investigators (Chesley & Chesley, 1939; Welsh, Wellen & Taylor, 1942) have asserted that in women pregnancy has no effect on renal function. More recent studies, however (Bucht, 1951; Brandstetter & Schüller, 1954; Dignam, Titus & Assali, 1958), have shown that there are progressive increases in both the glomerular filtration rate and the renal plasma flow during pregnancy, and that these changes reach a maximum in the third trimester and return to normal during the puerperium. This relationship appears to have been little studied except in man, and it therefore seemed worth while to find out whether similar changes occur in animals. Some of the results have already been published in a preliminary communication (Matthews & Taylor, 1959).

METHODS

Animals and diet. Virgin hooded rats of the Rowett strain, 3–4 months old and weighing between 200 and 300 g, were fed on the stock diet described by Thomson (1936), but differing from it in a higher content of skim milk (14% instead of 7%).

Mating. Male and female rats were placed together for a period of 16 hr, and the duration of pregnancy was timed from the day of mating.

Operative procedure. The method of Pritchard & Huggett (1947) was used for the removal of foetuses while leaving the placentas *in situ*, with the modification that the foetuses were removed from both uterine horns through a mid-line abdominal incision.

Anaesthesia. Pentobarbitone sodium B.P. (Veterinary Nembutal; Abbott Laboratories Ltd.; 5·5 mg/100 g body wt.) was given by intraperitoneal injection.

Determination of plasma inulin and para-aminohippurate (PAH) clearances. All methods used for collecting blood and urine from small animals have presented numerous difficulties (Smith, 1951) but despite this the results of many methods agree well. Dicker (1956) has pointed out that provided the urine collections are made while the concentration of inulin or PAH is changing quite slowly, the single subcutaneous injection technique will yield reasonably accurate results.

Inulin was supplied by Messrs Kerfoot and Co. Ltd., and the PAH obtained from L. Light and Co. Ltd. Two solutions, the pH of which was adjusted to approximately 7·0, were prepared for injection. One consisted of inulin 5 g/100 ml. and mannitol 5 g/100 ml. in 0·9% (w/v) NaCl solution. The other consisted of PAH 1·25 g/100 ml. in 0·9% (w/v) NaCl solution. Of inulin-mannitol solution, 1 ml./100 g body weight was injected. The amount of PAH solution to be injected was estimated from Friedman's dosage curve (Friedman, 1952) and varied between 4·0 and 4·5 ml. Ten minutes after the administration

of the anaesthetic these volumes of fluid, divided into four doses, were injected in rapid succession beneath the skin of the thorax and upper abdomen.

The routine procedure for determining the clearances was as follows: from the forty-eighth to the fiftieth minute after the final injection the bladder was washed out with at least 2 ml. of saline through an indwelling catheter and the washings discarded. Bladder urine was then collected from the fiftieth to the sixtieth minute, during the last 2 min of which the bladder was repeatedly washed and the washings added to the urine already collected. During the minute following the end of this period, approximately 3 ml. of blood was taken from the femoral vessels on one side into a tube containing a trace of heparin.

Analytical methods. Inulin in plasma and urine was determined by the method of Roe, Epstein & Goldstein (1949) and PAH in plasma and urine was estimated by the method of Bratton & Marshall (1939).

RESULTS

Three different experiments were made.

Experiment 1. Inulin and PAH clearances were measured in two groups each of twenty-four female rats. The first group consisted of unmated control animals and the second of pregnant animals mated 18 days earlier. The results for inulin and PAH clearances are given in Table 1 and show that both clearance rates were increased in the 18-day pregnant group when compared either absolutely, or per gram body weight, or per gram kidney weight, with the mean values for the unmated control group. These differences in the inulin and PAH clearances are significant ($P < 0{\cdot}001$ and $< 0{\cdot}05$, respectively).

TABLE 1. Mean values of inulin and PAH clearances in unmated control and 18-day pregnant anaesthetized rats

Clearance	Unmated control rats	18-day pregnant rats	Difference ± S.E.
Inulin			
ml./min	1·45	2·48	1·03 ± 0·083
ml./min/100 g body wt.	0·58	0·86	0·28 ± 0·024
ml./min/g kidney wt.	0·71	1·13	0·42 ± 0·044
PAH			
ml./min	5·87	7·74	1·87 ± 0·412
ml./min/100 g body wt.	2·35	2·65	0·30 ± 0·132
ml./min/g kidney wt.	2·86	3·52	0·66 ± 0·191

Experiment 2. In order to determine the effect, if any, of the stage of pregnancy, inulin and PAH clearances were measured in three further groups. The first group consisted of fourteen unmated control animals, the second of fifteen pregnant animals mated 14 days earlier, and the third of seventeen pregnant animals mated 21 days earlier. The figures in Table 2 show that in the 14-day pregnant rats both the inulin and PAH clearances were increased significantly when compared with the unmated controls or with the 21-day pregnant group ($P < 0{\cdot}001$). The mean inulin clearance rates for the 21-day pregnant rats were slightly but significantly greater ($P < 0{\cdot}02$) than the values for the controls except when the comparison

was made in terms of body weight, in which case the difference was not significant. The mean PAH clearances for the 21-day pregnant rats were somewhat less than the corresponding values for the control group but significant ($P < 0.01$) only when the measurements were made in terms of body weight.

TABLE 2. Mean values of inulin and PAH clearances in (*A*) unmated control, (*B*) 14-day pregnant, and (*C*) 21-day pregnant anaesthetized rats

Clearance	(*A*) Unmated control rats	(*B*) 14-day pregnant rats	(*C*) 21-day pregnant rats	Difference ± S.E. (*B*) − (*A*)	Difference ± S.E. (*B*) − (*C*)
Inulin					
ml./min	1·64	2·64	1·93	1·00 ± 0·073	0·71 ± 0·073
ml./min/100 g body wt.	0·63	0·92	0·66	0·29 ± 0·033	0·26 ± 0·033
ml./min/g kidney wt.	0·84	1·42	0·98	0·58 ± 0·051	0·44 ± 0·051
PAH					
ml./min	5·86	8·48	5·44	2·62 ± 0·261	3·04 ± 0·261
ml./min/100 g body wt.	2·26	2·95	1·86	0·69 ± 0·112	1·09 ± 0·112
ml./min/g kidney wt.	3·00	4·54	2·77	1·54 ± 0·170	1·77 ± 0·170

TABLE 3. Mean values of inulin and PAH clearances in anaesthetized rats in which (*A*) pregnancy was terminated, and (*B*) placentas were retained

Clearance	(*A*) Pregnancy terminated	(*B*) Placentas retained	Difference ± S.E.
Inulin			
ml./min	1·58	2·18	0·60 ± 0·071
ml./min/100 g body wt.	0·57	0·75	0·18 ± 0·019
ml./min/g kidney wt.	0·78	1·09	0·31 ± 0·037
PAH			
ml./min	5·01	7·02	2·01 ± 0·344
ml./min/100 g body wt.	1·80	2·41	0·61 ± 0·110
ml./min/g kidney wt.	2·49	3·53	1·04 ± 0·175

Experiment 3. In this experiment two groups of pregnant rats were used. The first consisted of sixteen animals in which pregnancy was terminated on the 13th day by removing the entire uterine contents. In the second group of seventeen, all foetuses were removed on the 13th day but the placentas were left *in situ*. Inulin and PAH clearances were then determined in both groups 5 days later, the 18th day after mating. Table 3 shows the results. In the group of rats in which the placentas were retained within the uterus after removing the foetuses, both the inulin and PAH clearances were greater than in those animals in which pregnancy was terminated by removing the entire uterine contents. These differences are significant ($P < 0.001$).

DISCUSSION

These results show that in the rat the course of pregnancy is accompanied by alterations in the plasma clearance rates of inulin and PAH. By the end of the second week of pregnancy both inulin and PAH clearances were considerably increased, and remained elevated on the 18th day. The inulin clearance showed an increase which was proportionately greater than the PAH clearance, with the result that on both the 14th and 18th days of pregnancy the filtration fraction was greater than that of the unmated control animals. On the 21st day clearance rates of both inulin and PAH were found to be lower than those obtained on the 14th and 18th days of pregnancy. The inulin clearance per gram of kidney weight on the 21st day was somewhat greater than that of the unmated controls, while the reverse held for PAH, with the result that the filtration fraction remained elevated. It seems therefore that pregnancy in the rat is accompanied for a period by increased plasma inulin and PAH clearances, which tend to fall towards the end of gestation, the rate of fall being more rapid for PAH than for inulin.

It is of interest to compare these results with changes in the inulin and PAH clearances found in normal human pregnancy. Bucht (1951) and Sohar, Scadron & Levitt (1956) found, early in human pregnancy, increased inulin and PAH clearances which reached a peak at the eighth month, the filtration fraction, however, remaining unchanged. Thereafter the PAH clearance fell to the control level while the inulin clearance remained raised. As a result the filtration fraction during the last month of pregnancy was greater than that for the first eight months. The relationship between pregnancy and renal clearance in other animal species does not seem to have been studied and comparison with the present results is not possible.

The results of the third experiment suggest that the placentas are in some way involved in the increased clearances found during pregnancy in the rat. Some of the bodily changes occurring during pregnancy are independent of the presence of the foetuses and there is evidence (Brooksby & Newton, 1938) that the maintenance of the increased body weight of pregnant mice requires the presence of living placental tissue. A similar finding has been reported by Campbell, Innes & Kosterlitz (1953) in respect of the rat. The actual mechanism by which the placentas bring about the increased clearances of inulin and PAH during pregnancy is at present being investigated.

SUMMARY

1. The effects of pregnancy on inulin and para-aminohippurate (PAH) clearances have been investigated in the anaesthetized rat.

2. Both inulin and PAH clearances were significantly increased in 18-day pregnant rats when compared with unmated control rats.

3. Similar increases were found in 14-day pregnant rats, but not in a group of 21-day pregnant rats.

4. Removal of the foetuses on the 13th day of pregnancy, leaving the placentas *in situ*, gave higher clearance values on the 18th day than when both foetuses and placentas were removed.

We are indebted to Mr J. F. Ingram for technical assistance.

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THE CONTRIBUTION OF THE INTERCOSTAL MUSCLES TO THE EFFORT OF RESPIRATION IN MAN

By A. TAYLOR*

From the Chemical Defence Experimental Establishment, Porton Down, Wiltshire

(*Received* 15 *January* 1960)

Though the diaphragm undoubtedly performs much of the work of normal breathing, the arrangement of the intercostal muscles suggests that they may also contribute to a greater or less extent. In a recent monograph, Campbell (1958) has summarized the various conflicting ideas that have been offered in the past on the functions of the intercostals and has shown that anatomical considerations alone are not capable of deciding either the precise mechanical actions or the extent to which such mechanically feasible actions of the muscles occur naturally. The same author (Campbell, 1955) has made electromyographic observations with surface electrodes and pen oscillograph and concluded that some parts of the intercostal muscles were active in quiet inspiration and in voluntarily forced expiration. No distinction was possible between the internal and external layers. Jones, Beargie & Pauly (1953) and Jones & Pauly (1957) have examined the intercostals in the parasternal and mid-clavicular regions with similar techniques and have stated that no rhythmic activity is to be found in the intercostal muscles in quiet breathing. Though their methods did not allow separate recording from the different layers, they nevertheless implied that the sole function of the intercostals was to maintain a constant distance between the ribs. Other workers interested in intercostal function, notably Tokizane, Kawamata & Tokizane (1951), Koepke, Murphy, Rae & Dickinson (1955) and Nieporent (1956) have employed needle electrodes and oscilloscopic display, but were still unable to record separately from the various layers. However, in view of the evidence of Draper, Ladefoged & Whitteridge (1957) that the internal intercostal muscles might act independently of the external, at least in speech, it was thought worth while to study the subject again with more precise electromyographic technique.

The following observations were therefore made on a number of normal human subjects to determine, first, whether the action of the different

* Present address: Department of Physiology, King's College, London, W.C. 2.

layers of intercostal muscle could be recorded separately, and secondly, the circumstances of this contraction in various parts of the chest wall.

METHODS

Electromyography. In an attempt to locate activity detected in the intercostals by surface electrodes, Pauly, Jones, Jarach & Totman (1957) have devised an ingenious 'vector-electromyographic' technique. Though this method may give information on the depth and arrangement of active motor units, its interpretation is uncertain. It was preferred in the present work to use a more direct approach with needle electrodes of special construction.

The requirements in the design of suitable electrodes are first that they shall be as fine as possible to minimize discomfort and secondly that they shall have a short range so as to record separately from closely adjacent muscles. The second requirement is satisfied by the use of a bipolar electrode and a differential amplifier with high in-phase discrimination. Such a system was employed by Bigland & Lippold (1954) to record from a small number of motor units in strongly contracting muscle and by Faarborg-Andersen (1957) in a study of the laryngeal muscles. Bipolar electrodes had the added advantage in the present work of discriminating against interference from the electrical activity of the heart and from the 50 c/s mains supply.

Needle electrodes were made of specially fine tubing to satisfy the first requirement and also, incidentally, to shorten the pick-up range still further. Two 50 μ enamelled platinum wires were cemented with 'Araldite' inside thin-walled stainless steel tubing of outside diameter 0·30 and 0·15 mm bore, which was finally sharpened to a 30° bevel. These fine needles retained their position in actively contracting muscle better than did those of thicker gauge; but even so, movement artifact was sometimes troublesome because of the very short range of the electrodes. Consequently, some of the records were made with a unipolar needle. Naturally, the volume of muscle from which activity was detectable was very much greater than with the bipolar electrodes, but the unipolar needles were only used after exploration with the more selective ones had shown the type of activity to be expected. Two channels of differential a.c. amplification were provided for the electromyograms which were displayed on a twin-beam oscilloscope and monitored by loudspeakers.

Respiration. Breathing was recorded, in the earlier experiments, by means of a closed-circuit bell spirometer, the movement of which operated one beam of a second oscilloscope. However, the spirometer was inconvenient, was not capable of showing rapid changes in rate of air flow and might, by its inertia and the resistance of the connecting tubes, have added to the muscular effort required for respiration. Consequently, the spirometer was later replaced by a screen pneumotachograph operating a sensitive R.C.A. 5734 transducer manometer, which gave records of air-flow rate directly on one beam of the oscilloscope and of volume, by electronic integration, on the other. All the traces were photographed together on continuously moving bromide paper.

Procedure. The subjects were 80 young male service volunteers with no clinical or radiological evidence of chest disease. The observations were made with the subject lying supine on a tilting table with a thin pillow under his head. The table had a foot rest and could be tilted to bring the subject to within 10° of the erect posture with the minimum of disturbance and activity on his part. The back of the chest was made accessible by way of a slot cut through the table. For convenience, some of the observations on the back were made with the subject lying on his left side or seated with his arms folded on his knees.

Intercostal muscles. The initial experiments, designed to see whether the layers of intercostal muscle could be shown to act separately, were performed as follows. An electrode was introduced through the skin in the chosen site and advanced until action potentials were first detected in quiet breathing, forced breathing, coughing or speech. Confusion with overlying muscles such as the pectorals and serratus anterior was expected but in practice

was easily tested for by having the subject make movements which employ these muscles and advancing the needle point until the unwanted activity was eliminated. Records of the characteristic intercostal activity were then made and the needle moved on until a changed pattern of activity appeared or the needle point had reached the pleura. In this way all the accessible parts of the intercostals were explored. The danger of penetrating the pleura was very much less than it may appear. Once the needle had entered the intercostal muscle, great care was exercised, and usually a narrow zone with no activity was noticeable after penetrating the muscle, and before the pleura was touched. Withdrawal at this stage avoided causing pleural pain, the occasional occurrence of which was, in any case, the signal for prompt withdrawal. Only one of the subjects suffered any untoward effect, in the form of a small pneumothorax, which was quickly reabsorbed.

During the course of these initial explorations and as the subject of later experiments, observations were made to determine what parts of the intercostal muscles were active under conditions of normal quiet breathing in the supine position. Care was required to ensure that the subject was quite relaxed and comfortable, otherwise continuous discharge in the tense muscles spoiled the observations. On the other hand, some men, anxious to co-operate, exaggerated their respiratory movements unless they were carefully instructed to relax and given plenty of time to get used to the procedure. Care was needed in the use of the monitoring loudspeakers as some subjects tended to overbreathe enough to maintain a rhythmic discharge which did not otherwise occur during quiet breathing. On each occasion, once the activity in quiet breathing had been determined, the effect was tried of voluntary deeper breathing, coughing and speech. In some experiments the effect of change of posture was tried by tilting the subject to the nearly erect attitude.

Diaphragm. The diaphragm has been studied repeatedly in animals but few observations have previously been reported on human subjects. Tokizane *et al.* (1951) referred to some electromyographic experiments in man but gave no details or recordings. Campbell (1958) has been unsuccessful in attempts to record with surface electrodes. Draper *et al.* (1957), using surface electrodes mounted on an oesophageal tube, have managed to record from the crural parts of the muscle. In the present work it was found very satisfactory to record from the diaphragm with needle electrodes inserted below the 9th or 10th ribs in the right mid-axillary line. Preliminary X-ray tomograms were made to determine the lower limit of the costodiaphragmatic recess in the mid-axillary region. Since this method was employed an essentially similar one has come to notice, used by Nieporent (1956).

RESULTS

In each of the 80 subjects several sites were explored. Thirty-five of the men were used on one occasion only, the rest two or three times. In all, about 250 observations with needle electrodes were necessary to learn the technique and to form some idea of the range of individual variation.

Two layers of intercostal muscle. Two functionally distinct layers of intercostal muscle were demonstrated in all parts of the chest wall with the exceptions of the interchondral region anteriorly and the area medial to the angles of the ribs posteriorly, in which parts only single layers were evident. Where two layers existed, the superficial one (external intercostals) was activated only by inspiratory efforts, and the deeper one (internal intercostals) by expiratory efforts. This finding is illustrated in Fig. 1 (*a*) and (*b*).

The interchondral muscles of the upper four or five spaces (subsequently

referred to as the parasternal region), though anatomically part of the internal intercostals, were active nevertheless only during inspiration (Fig. 2 (*a*)). The interchondral muscles of the lower spaces, on the other hand, contracted in expiratory efforts like the internal layer elsewhere (Fig. 2 (*b*)). The external intercostal muscle, where it existed as a single layer, medial to the angles of the ribs posteriorly, was found to function in inspiration as it did in other regions where it was the outermost of two layers.

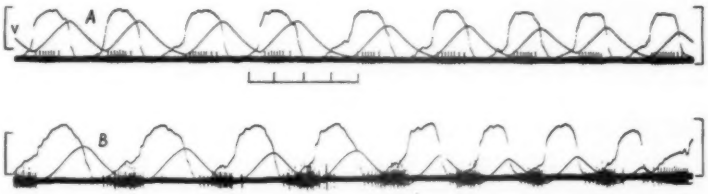

Fig. 1. Exploration of the full thickness of the chest wall in the 3rd right intercostal space in the mid-clavicular line (3 RICS, MCL). *A*, needle tip in superficial layer detects expiratory activity. *B*, needle tip in deeper layer detects expiratory activity. Calibration: left hand, volume record 1 l.; right hand, pneumotachogram, 20 l./min; time scale, seconds. In each record the smooth curve shows the volume, *V*.

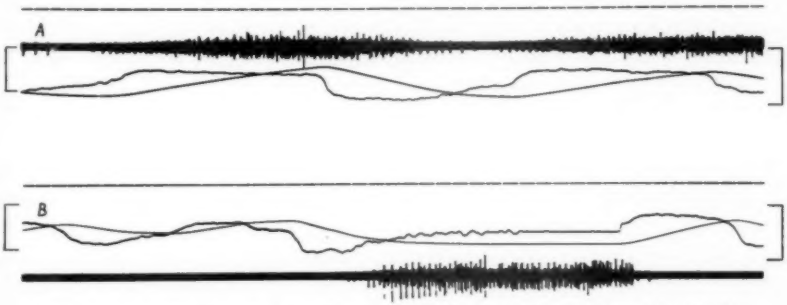

Fig. 2. *A*, inspiratory activity in parasternal internal intercostal muscle (3 RICS). *B*, expiratory activity in internal intercostals (5 RICS, MCL) during a forced expiratory effort against the closed glottis. Calibrations: volume and pneumotachogram as in Fig. 1; time scale, uppermost trace interrupted three times a second.

The parasternal region, where the internal intercostals appeared to have an inspiratory function, was explored in more detail in nine subjects with the object of determining its lower limits. The lowest intercartilaginous space in which the muscles were inspiratory was the third in two subjects, the fourth in five and the fifth in two. During the course of these experiments expiratory activity was sometimes noticed when the needle was entered a little more deeply than usual in the parasternal region and it was

suspected that this might have come from the sterno-costal muscle. Subsequently, it was found possible to record regularly from this muscle in the second, third and fourth intercartilaginous spaces, where it was active in expiratory efforts in the same way as the internal intercostal muscles elsewhere.

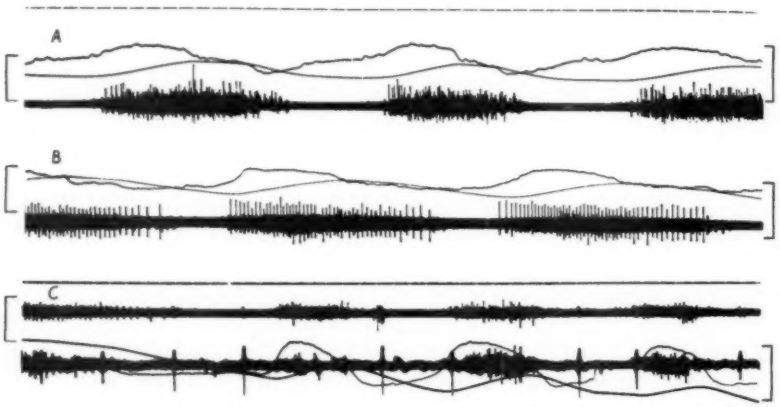

Fig. 3. Activity of the diaphragm during normal, quiet breathing, recorded with needle electrodes at the right costal margin. *A*, recorded with unipolar needle. *B*, recorded with fine bipolar electrode. *C*, simultaneous recordings through a needle electrode at the costal margin (above) and through oesophageal electrodes (below). Calibrations as in Fig. 2.

Muscular activity in quiet breathing

The diaphragm. Figure 3 (*a*) shows the activity recorded with a unipolar electrode inserted into the edge of the diaphragm. Action potentials commenced immediately before inspiratory airflow, increased rapidly to a maximum and died away in the first half of the expiratory phase. A similar recording made with a fine bipolar needle is shown in Fig. 3 (*b*), in which the activity of a single unit is obvious.

With five subjects attempts were made to record from the diaphragm with surface electrodes mounted on an oesophageal tube and simultaneously with needles inserted at the costal margin. It was technically difficult to secure good oesophageal recordings because e.c.g. interference was troublesome and sudden changes in electrode position occurred due to swallowing movements. Figure 3 (*c*) illustrates the type of record obtained, from which it is seen that there is no real difference in the timing of the activity detected in the two sites.

Intercostals. During quiet breathing, rhythmic activity was evident only in certain limited parts of the intercostals. The intercartilaginous internal intercostals of the parasternal region were regularly active during inspira-

tion, even in the quietest breathing. Thus Fig. 4 (*a*) shows simultaneous records of contraction in the parasternal part of the third right intercostal space (3 RICS) and in the diaphragm during a tidal respiratory excursion of only 300 ml. Elsewhere there was seldom any detectable activity of the intercostals in inspiration under these conditions.

Expiratory activity in the intercostal muscles occurred in the internal layer only, and was limited, during quiet breathing, to the lower four spaces between the anterior axillary line and the angles of the ribs. A typical record from the internal intercostal muscles of the 8 RICS in the mid-axillary line is shown in Fig. 4 (*b*), with the reciprocal activity in the diaphragm.

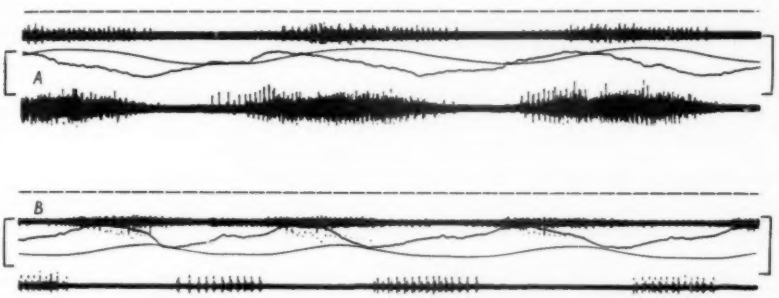

Fig. 4. *A*, inspiratory activity of parasternal internal intercostal (3 RICS, upper trace) and of diaphragm (lower trace) in quiet breathing (subject supine). *B*, expiratory contraction of internal intercostals in 8 RICS in the MCL (upper trace) and inspiratory contraction of the diaphragm (lower trace) in quiet breathing. Calibrations as in Fig. 2.

It was not possible to confirm, with bipolar needle electrodes, the occurrence of inspiratory activity as reported by Campbell (1955, 1958) in the lower intercostal spaces by means of surface and unipolar needles. On the contrary, the only activity in these lower spaces during quiet breathing was found in the internal layer at the side of the chest, and that in the expiratory phase. It was thought that the activity detected by Campbell in inspiration might have been that of the diaphragm. The peripheral parts of this muscle lie in close contact with the inner aspect of the lower ribs and might have been within the range of surface electrodes or unipolar needle electrodes. Accordingly, an attempt was made to see whether this could have been so by recording simultaneously with surface and bipolar needle electrodes in the seventh right intercostal space in the mid-clavicular line. With the subject breathing quietly in the supine position, the needle in the intercostal (a single layer at this point) showed no activity, while the surface electrodes picked up distant inspiratory discharges. In deep breathing the needle revealed marked expiratory

activity only. The surface electrodes, however, showed activity in both phases, that in inspiration being clearly from a much more distant source (diaphragm) than that in expiration (intercostals). The same results were obtained with an ink-writing electroencephalographic (e.e.g.) machine like that used by the workers quoted above, except that the fast action potential components detected by the bipolar electrodes were relatively much more attenuated by the machine than were the slower, integrated potentials from the surface electrodes.

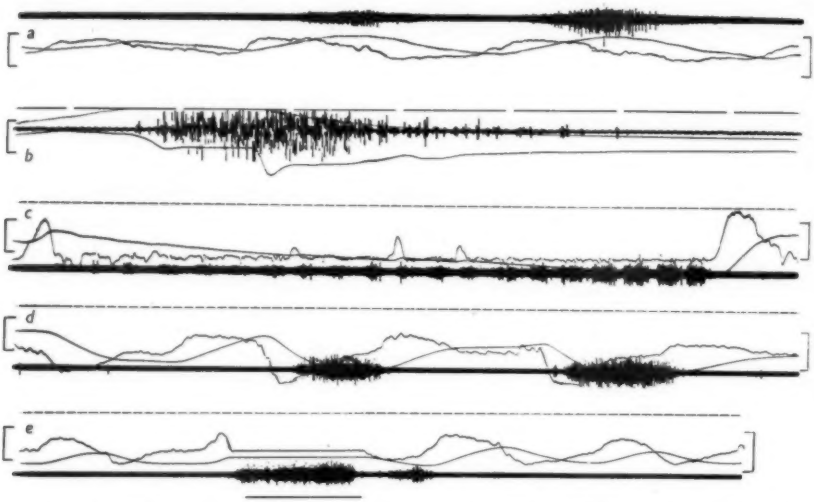

Fig. 5. *a*. Recruitment of external intercostal activity (9 RICS, just medial to angle of ribs) in voluntary overbreathing. *b*. Activity in the internal intercostal muscle (6 RICS, MCL) during a voluntary cough. *c*. Action of the internal intercostals during speech (counting from 1 to 20 with a single breath). *d*. Expiratory activity in the internal intercostals (7 RICS, anterior axillary line) during voluntary overbreathing. *e*. Activity in the internal intercostal muscle (6 RICS, MCL) during Valsalva's manoeuvre. Calibrations as in Fig. 2.

Muscular activity in more vigorous respiratory efforts

Though, as pointed out above, the external intercostal muscles did not appear to contribute to the effort of quiet breathing they were readily recruited by deeper inspirations taken either voluntarily or as a result of rebreathing from a bag of oxygen. This is illustrated in Fig. 5 (*a*) for the muscles of the 9 RICS just medial to the angles of the ribs, and similar results have been obtained in all other parts of the chest wall examined.

Similarly, the internal intercostal muscles showed only very restricted activity in quiet breathing, but were brought into action all over the chest (except in the parasternal region) by more vigorous expiratory

efforts such as coughing, speech, forced expiration and the Valsalva manoeuvre (Fig. 5 (*b*)–(*e*)). In the parasternal region the sternocostal muscle was active in these circumstances. Internal intercostal muscles in regions not normally active in quiet breathing, were sometimes briefly active on resuming normal breathing after a forced expiratory effort against the closed glottis. This is illustrated in Fig. 5 (*e*).

The effect of change of posture

By means of the tilting-table arrangement described above, it was possible to record the purely respiratory action of the intercostals of the subject in the almost erect position. Provided the man could relax fully, there was no continuous contraction due to the use of the muscles in supporting the trunk and any discharges which occurred were rhythmically related to respiration. No differences in pattern of action of the intercostals or diaphragm were found in the erect subject from that detailed above for the supine one.

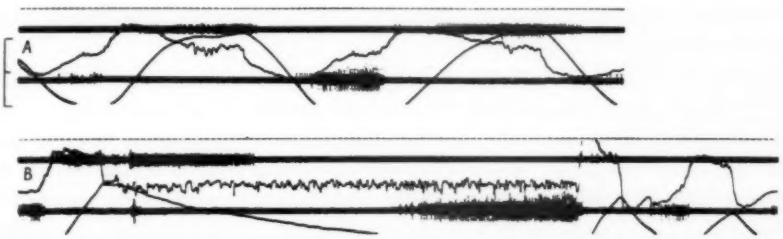

Fig. 6. Reciprocal activity in the intercostal muscles (3RICS, MCL). Upper electromyogram trace, external layer; lower trace, internal layer. *A*, voluntary overbreathing; *B*, counting 1–20 with one breath. Calibrations as in Fig. 2.

Reciprocal activity of the layers of intercostal muscle

It was noticed that, under conditions of quiet breathing, the intercostals contributed little to the effort of respiration and that the active parts of the inspiratory outer layer and the expiratory inner layer did not overlap, so that attempts to demonstrate reciprocal action in quiet breathing were not successful. However, in more vigorous efforts, when the intercostals exhibited their characteristic activities all over the chest wall, rhythmic reciprocal action of the two layers did occur, and is illustrated in Fig. 6.

DISCUSSION

The above results have shown that, provided the electromyographic technique is sufficiently precise, it is possible to record separately the activities of the internal and external intercostal and sternocostal muscles,

and that it is possible and safe to record from the diaphragm with needle electrodes in the conscious human subject.

The idea that the external intercostals are inspiratory and the internal expiratory was put forward long ago by Hamberger (1848) and has been supported in animal experiments by Bronk & Ferguson (1934), Anderson & Lindsley (1935), Gesell (1936), Hukahura, Okada & Yamagami (1957) and Chennels (1957). The present results show that a similar situation obtains in the human subject and makes it likely that the discordant results obtained on man in the past have been due to the use of techniques not capable of separating activity in the internal and external intercostal muscles. It has been found in the present experiments that in regions of the chest wall where two layers of intercostal muscle exist, only a very small movement of the needle tip was necessary to pass from one type of activity to the other. For this reason the fine bipolar needle electrodes of very short range must be used if the activity of the two layers is to be distinguished. The range of the electrodes employed in this work must have been less than the 0·5 mm found by Krnjević & Miledi (1958) for wires separated by 0·19 mm, since in the present case the wires were rather closer (0·10 mm). While these electrodes could usually be relied upon to retain their position in the muscle during quiet breathing, they were often quickly dislodged by stronger efforts. This explains the difficulty found in recording the reciprocal actions of the internal and external layers simultaneously. There appears to be no region where reciprocal action occurs during quiet breathing, and forced breathing (during which reciprocal action does occur) tends to dislodge one or other of the electrodes. Movement of the needles with respiration is least troublesome in the upper part of the front of the chest. It was from this part that the record of reciprocal activity in Fig. 6 was obtained.

The fact that intercostal action in quiet breathing is limited to the parasternal region in inspiration and the lower lateral part in expiration suggests that there may be some peculiarity in the mechanical action of the thoracic cage in these two parts. Of interest in this respect is Keith's (1904) proposal that the thorax functioned as three parts; namely, an 'operculum' composed of first rib and manubrium, an antero-lateral part composed of sternum and true ribs, and the floating ribs which he regarded as functionally part of the abdominal wall. Campbell (1958) has emphasized that an important mechanical action of the diaphragm is to expand the lower part of the chest. This action results from the fact that the diaphragm's lateral fibres, where they arise from the costal margin, are almost vertical. Their contraction thus elevates the ribs which, by their bucket-handle action, expand the thorax laterally. The anterior fibres of the diaphragm, on the other hand, being nearly horizontal must tend to

depress the sternum on the manubriosternal joint. The action of the parasternal internal intercostal during inspiration is likely to oppose this tendency.

It is interesting to find that expiration, even in quiet breathing, is not entirely passive. In the first place, the diaphragm and parasternal muscle do not relax abruptly at the end of inspiration, but gradually during the first half of the expiratory phase. Secondly, contraction of the internal intercostal muscle at the lower lateral part of the chest appears to represent an active expiratory effort. Why this region alone should be involved is not clear. Perhaps elastic recoil of the thin wedge of lung in the costo-diaphragmatic recess might otherwise be inadequate for the proper ventilation of this part.

The innermost layer of muscle of the chest wall is generally referred to as the transversus thoracis, as it is the thoracic equivalent of the transversus abdominis. It is an incomplete layer comprising three parts, namely the sternocostal anteriorly, the intercostales intimi at the side of the chest and the subcostales posteriorly. The action of the sternocostal muscle has been shown above to be expiratory. In the present observations on the lower lateral part of the chest wall (including those for recording from the diaphragm) exploration with the needle in front of the anterior edge of the external intercostal muscle never showed evidence of (functionally) more than one layer of thoracic muscle. Instead, expiratory activity alone was noted, although the full thickness of the chest wall had undoubtedly been penetrated. This makes it likely that the intercostales intimi, which must have been traversed by the needle, are purely expiratory in function. These muscles overlap the internal intercostal at this point, so that presumably the expiratory muscle layer here is of some importance. Only a small number of observations were made on the back of the chest and no definite evidence was obtained of the action of the subcostales. However, it seems likely by analogy with the other parts of the transversus thoracis and by virtue of their anatomical arrangement that they also function as expiratory muscles. In this event the innermost layer of thoracic muscles appears to be developed only in those regions where the intercostal muscles, internal or external, are deficient, and to act like the internal intercostals elsewhere, namely as expiratory muscles. Figure 7 shows diagrammatically the findings in the present investigation.

What are the mechanical implications of the existence of two layers of muscle all over the chest wall, with the external always inspiratory and the internal always expiratory? There are two factors in the anatomical arrangement of muscles which determine their mechanical action, namely their points of attachment and the direction of their fibres. The fibres of the external intercostal muscles are almost horizontal, so that their

contraction will tend to draw a particular rib laterally (acting in the front of the chest) and backwards (acting at the side) with respect to the rib above. This simple action of the external intercostals is complicated by the fact that their fibres do not lie in the curved surface defined by the axes of the ribs (i.e. the middle of the musculo-skeletal layer of the chest wall) but are attached external to it. They must, therefore, exert a rotational

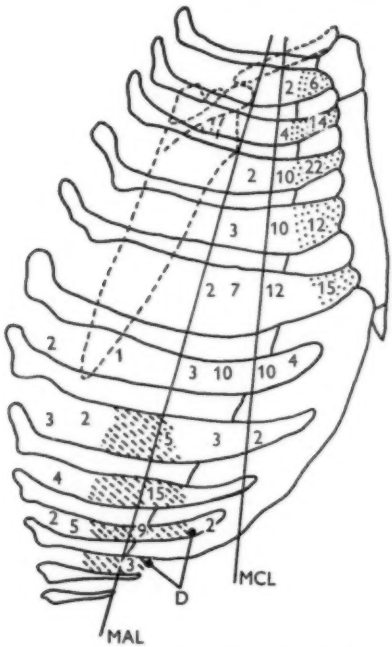

Fig. 7. Diagrammatic projection of the right side of the chest to show where intercostal activity was found in quiet breathing (dotted area, inspiratory; hatched area, expiratory). The numbers indicate the numbers of observations made in each site. The two points marked D indicate where electrodes were placed for recording from the diaphragm.

couple on each rib tending to evert it on the one above. It is clear that such an action helps to expand the chest and opposes the tendency of the negative pressure generated within the thorax by the diaphragm to collapse the chest wall.

Similarly, the contraction of the almost vertical fibres of the internal intercostal muscle during expiration is made more effective in compressing the chest by the fact that the muscle is attached near the inner aspect of the ribs and must consequently exert a turning couple on them tending to invert each rib on the one above. It is particularly noteworthy that

though the direction of the fibres of the internal intercostal muscle is the same in the parasternal region as elsewhere, nevertheless, the parasternal part has an inspiratory function rather than the usual expiratory. This suggests that the anatomical difference which determines an inspiratory function for the parasternal part may be that it has here become the outer layer (the inner layer being the sternocostal muscle).

SUMMARY

1. The respiratory functions of the intercostal muscles in human subjects have been investigated by electromyographic techniques capable of distinguishing the activity in the various layers. Simultaneous records have been obtained from the edge of the diaphragm for comparison with those from the intercostals.

2. The external intercostal muscle is purely inspiratory in action, whereas the internal layer is an expiratory muscle with the exception of its parasternal part, which is inspiratory.

3. The transversus thoracis contributes to expiratory efforts and is well developed only in those regions where there is a deficiency in one or other of the layers of intercostal muscle.

4. In fully relaxed subjects, breathing quietly in the supine position, activity occurs only in two restricted parts of the intercostal muscles; namely, the internal intercostal in the parasternal region during inspiration and in the lower, lateral part of the chest during expiration.

5. More vigorous respiratory efforts bring the layers of muscle into reciprocal action all over the chest wall, the external layer being inspiratory and the internal layer expiratory.

6. The significance of this arrangement is discussed and it is suggested that the inner and outer layers of muscle can exert opposite rotational forces on individual ribs.

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